EXPRESION Y DISECCION FUNCIONAL IN VITRO DE LAS HORMONAS LACTOGENICA PLACENTARIA Y DEL CRECIMIENTO HUMANO.

TESIS
QUE EN OPCION AL GRADO DE

DOCTOR EN CIENCIAS
CON ESPECIALIDAD EN BIOLOGIA MOLECULAR
E INGENIERIA GENETICA

PRESENTA

M.C. DIANA RESENDEZ PEREZ

MONTERREY, N.L. DICIEMBRE, 1991

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POR

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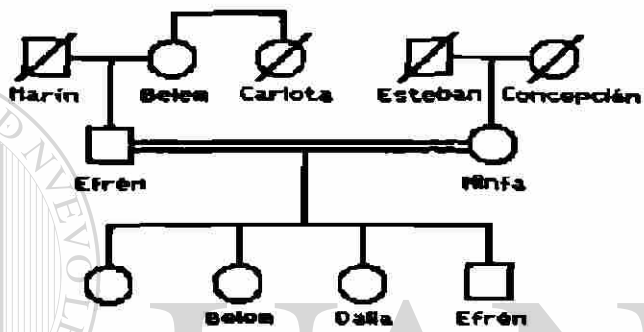
PRESENTA

M.C. DIANA RESENDEZ PEREZ

El presente trabajo se realizó en la Unidad de Laboratorios de Ingeniería y Expresión Genéticas (ULIEG), del Departamento de Bioquímica, de la Facultad de Medicina, de la Universidad Autónoma de Nuevo León en Monterrey; bajo la dirección del Ph.D. Hugo A. Barrera Saldaña.

DEDICATORIA

A quienes han sido y seran siempre mi adoración:



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*Se emplea aquí el término in vitro para referirse a estudios realizados en cultivo celular, e in vivo para aquellos efectuados directamente en el organismo, órgano o tejido.

LISTA DE ABREVIATURAS

CAT	Cloranfenicol acetil transferasa
dCTP	Desoxicitidina trifosfatada
DNAc	DNA complementario al RNA mensajero
DHFR	Dihidrofolato reductasa
hCMV	Citomegalovirus humano
h	Hora (s)
hGH	Hormona del crecimiento humano
hPL	Hormona lactogénica placentaria
KDa	Kilodaltones
Kpb	Kilopares de bases
LTR	Repeticiones terminales largas
M	Concentración molar
MEM	Medio esencial mínimo de Eagle
MT	Metalotioneína
μCi	Microcuries
mg	Miligramos
ml	Mililitros
μg	Microgramos
μl	Microlitros
min	Minuto (s)
N	Concentración normal
pb	Pares de bases
Prl	Prolactina
pH	Logaritmo negativo de la concentración de iones H^+
RIA	Radioinmunoensayo
RNAm	RNA mensajero
RNAshn	RNAs heterogéneos nucleares
SDS	Dodecil sulfato de sodio
SFT	Suero fetal de ternera
SMC	Sitio múltiple de clonación
SV40	Virus del simio 40
VSM	Virus del sarcoma murino

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AGRADECIMIENTOS

Me gustaría agradecer a todas las personas que hicieron posible el desarrollo y culminación de este proyecto. Si intentara citar sus nombres, incluso en orden alfabético, temo que no podría reconocer con justicia la ayuda y apoyo de todos mis maestros, colegas, compañeros, estudiantes y amigos porque no sabría donde trazar la línea y lo que es peor, podría olvidar a alguno de ellos. Las contribuciones fueron numerosas, diversificadas y van desde individuales hasta institucionales y de colaboración por lo que me resisto a ordenar sus esfuerzos segun estos criterios ya que todas fueron muy valiosas, sin excepción.

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RESUMEN

Los genes que codifican para la hormona del crecimiento (hGH) o somatotropina y para el lactógeno placentario (hPL) o somatomamotropina coriónica (hCS) constituyen en el humano un complejo multigénico localizado en las bandas q22-24 del cromosoma 17. El complejo multigénico hGH-hPL contiene dos genes para hGH y tres genes para hPL. Los productos de expresión mejor conocidos de estos genes son hormonas polipeptídicas de 22 Kilodaltones (KDa). Estas hormonas se sintetizan en diferentes tejidos bajo diferentes mecanismos de control.

Como un paso necesario para la realización del presente trabajo primeramente construimos y seleccionamos los vectores mas eficientes para la expresión de genes eucarióticos en cultivo de células y diseñamos un sistema para detectar proteínas secretadas como producto de la expresión de los genes introducidos transitoriamente a células en cultivo. Una contribución más de nuestro trabajo fué el desarrollo de una estrategia para cuantificar la expresión génica in vitro. Esta consistió en el intercambio de secuencias nucleotídicas entre los genes hGH y hPL; por lo que la denominamos mutagénesis por DNA homólogo.

En este trabajo establecimos las condiciones para detectar y cuantificar la expresión de hGH en cultivo celular. La actividad biológica de las hGHs producidas en cultivo celular fue verificada en el modelo de diferenciación de fibroblastos preadipocíticos hacia adipocitos. Una vez que estandarizamos las condiciones para la expresión funcional de hGH en nuestro modelo in vitro, determinamos que tanto hPL-3 como hPL-4 contribuyen a la producción de una misma hPL madura. La cuantificación de la expresión de los genes hPL-3 y hPL-4 a nivel de proteínas y RNA mostraron que la región codificante del gen hPL-3 presentó una mayor expresión que la del gen hPL-4. Interesantemente, encontramos que la abundancia de estas proteínas dependió del origen genético de los primeros dos exones. Ademas, las estrategias anteriormente descritas nos permitieron cuantificar el efecto de la mutación presente al inicio del segundo intrón del gen hPL-1. En estos experimentos detectamos una disminución total de la proteína codificada por el gen hPL-3 con esta mutación y no detectamos la reactivación de la expresión con el gen hPL-1 reparado.

Los resultados anteriores nos permitieron llegar a las siguientes conclusiones:

- Los genes hPL-3 y hPL-4 contribuyen a la producción de la hormona hPL madura en una relación de 4 a 1 en nuestro modelo in vitro.

- La expresión diferencial in vitro de los genes hPL-3 y hPL-4 depende de las diferencias nucleotídicas presentes en los dos primeros exones de estos genes.

- La mutación presente al inicio del segundo intrón del gen hPL-1 es una de las causas que afecta su falta de expresión.

El intercambio de secuencias homólogas entre estos genes nos permitió evaluar el efecto en la expresión de mutaciones sin modificación de la conformación nativa de la proteína codificada, produciendo así nuevas estrategias para el estudio de la expresión del genoma humano.

El modelo experimental que hemos desarrollado podría utilizarse en estudios de mutaciones existentes en genes que codifican para proteínas relacionadas genéticamente y para las que existen anticuerpos utilizando la conveniencia de la cuantificación de la proteína mediante RIA.

INTRODUCCION

El nacimiento de la tecnología del DNA recombinante a principios de la década de los 70's, marcó el inicio de una nueva era en Biología Molecular. Con la ayuda de esta tecnología ha sido posible iniciar el análisis, a nivel molecular, del altamente complejo genoma eucariote. Actualmente se estan llevando a cabo numerosos estudios con el propósito de analizar la estructura molecular y organización de genes para entender su función, regulación y origen.

La regulación de la expresión genética, implica la formulación de múltiples interrogantes y la consideración de los diferentes niveles por los cuales ésta información, depositada en un pequeño segmento cromosómico, pasa antes de alcanzar su manifestación final, reflejada en la estructura y función de la proteína en estudio.

La presencia de mutaciones en genes eucarióticos pueden afectar los niveles de su expresión debido a una alteración en los diferentes pasos de la ruta de expresión génica: transcripción, procesamiento del RNA, estabilidad del RNAm y traducción (1). Los efectos de mutaciones puntuales en la expresión de los genes estucturales estudiados requieren métodos analíticos altamente selectivos para detectar estos cambios.

Los genes que codifican para la hormona del crecimiento humano (hGH) o somatotropina y para el lactógeno placentario humano (hPL) o somatomamotropina coriónica (hCS) constituyen en el humano un complejo multigénico constituído por cinco genes (1-3). El aislamiento y caracterización de los genes del complejo multigénico han permitido estudiar su expresión y regulación. Los productos de expresión mejor conocidos de este complejo génico son los polipéptidos hGH y hPL de 191 aminoácidos, producidos en hipófisis y placenta respectivamente. Algunos polipéptidos codificados por estos genes son hormonas cuya función no ha sido descrita. Por lo que el complejo multigénico hGH-hPL es sin duda un modelo excelente para investigar los mecanismos moleculares mediante los cuales estos genes, con un alto grado de similitud, expresan hormonas relacionadas en tejidos diferentes.

I El complejo multigénico hGH-hPL.

El advenimiento de la tecnología del DNA recombinante y la clonación molecular de genes de

organismos superiores, ocasionaron una revolución en los laboratorios dedicados al estudio de la expresión y regulación genética. Los trabajos experimentales con el complejo hGH-hPL se iniciaron con el estudio de la forma madura de las proteínas y se prosiguió en la dirección del origen de la información genética responsable de la síntesis, estructura y función de estas hormonas.

A Localización cromosómica.

La localización subcromosómica de los genes fué determinada en estudios de hibridación in situ utilizando un fragmento del DNA complementario al RNA mensajero (DNAc) de hPL (ver sección IIA). Estos experimentos demostraron que los genes que codifican para hGH y hPL constituyen en el humano un complejo multigénico localizado en las bandas q22-24 del brazo largo del cromosoma 17 (3). Además, cuando los DNAc de hGH o hPL fueron utilizados para buscar secuencias específicas en bancos de DNA genómico humano, determinaron la presencia de cinco genes diferentes en el complejo hGH-hPL(1-3).

B Anatomía molecular.

El orden físico de los genes del complejo multigénico hGH-hPL fué determinado mediante digestión con enzimas de restricción de clonas sobrepuestas de fagos y cósmidos recombinantes conteniendo DNA humano y aislados por hibridación con sondas del DNAc de hGH o de hPL (1,2). Estos genes se encuentran arreglados partiendo del extremo 5' al 3'(ver figura 1) de la siguiente manera: primeramente hGH normal (hGH-N), luego hPL-1, también llamado hPL-like (hPL-L o hCS-L), posteriormente hPL-4 (también denominado hCS-A), al cual le sigue hGH variante (hGH-V) y finalmente hPL-3 (también designado como hCS-B). Los genes hGH y hPL presentan la misma orientación transcripcional y estan separados por regiones intragénicas de 6 a 13 kilopares de bases (kpb). Secuencias repetidas de la familia Alu fueron localizadas flanqueando los genes o en sus intrones (2,4 y 5). Las secuencias del extremo 5', regiones codificantes e intrones mostraron al menos un 90% de similitud entre hGH y hPL.

C Estructura génica.

El DNAc de hPL obtenido por Barrera-Saldaña y

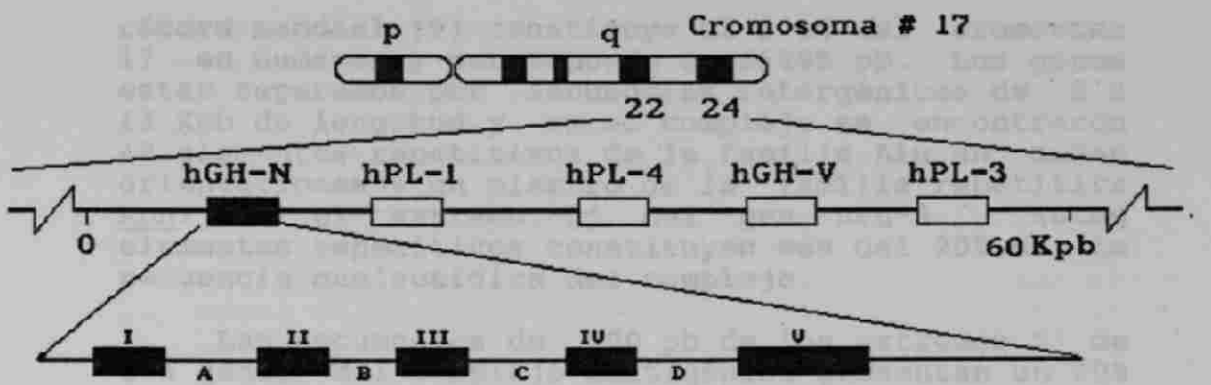

Figura 1. El complejo multigénico hGH-hPL. En este diagrama se presenta la localización cromosómica, anatomía molecular y estructura génica del complejo multigénico hGH-hPL.

col. en 1982 (ver sección IIA) fue hibridado con el gen hPL-3 para construir lo que se conoce como heteroduplex (6). El análisis al microscopio electrónico de estos heteroduplex reveló la presencia, en el gen para hPL, de cuatro intrones. El tamaño de los intrones y exones determinados en este estudio fueron similares a los descritos para el gen hGH por De Noto y col. en 1981 (7).

Estudios posteriores confirmaron que los cinco genes del complejo génico presentan la misma organización génica con cinco exones y cuatro intrones en la misma posición. El patrón de corte con enzimas de restricción permitió identificar cada uno de estos genes. Los dos genes hGH están contenidos en fragmentos EcoRI de 2.6Kpb. Ambos genes tienen sitios únicos para la enzima BglI, sin embargo difieren en la posición de sitios BamHI. Dos de los genes para hPL están contenidos dentro de fragmentos EcoRI de 2.9Kpb, presentan sitios únicos para las enzimas XbaI y BamHI y difieren en la presencia de uno (hPL-3) o dos (hPL-4) sitios PvuII. El tercer gen hPL-1 que contiene secuencias relacionadas con hPL está contenido en un fragmento EcoRI-XbaI de 3.5Kpb que no presenta sitios de corte con las enzimas de restricción XbaI ni BamHI.

D Secuencia nucleotídica.

Un extraordinario esfuerzo fué realizado por Chen y col., en 1989 cuando determinaron la secuencia nucleotídica completa del complejo multigénico hGH-hPL (8). Esta secuencia considerada

récord mundial (9) constituye el 0.1% del cromosoma 17 en humanos y corresponde a 66495 pb. Los genes estan separados por secuencias intergénicas de 6 a 13 Kpb de longitud y en el complejo se encontraron 48 elementos repetitivos de la familia Alu en ambas orientaciones y un miembro de la familia repetitiva KpnI en el extremo 5' del gen hPL-3. Estos elementos repetitivos constituyen más del 20% de la secuencia nucleotídica del complejo.

Las secuencias de 500 pb de los extremos 5' de los genes del complejo multigénico presentan un 90% de similitud en sus secuencias nucleotídicas. Esta similitud se extiende hasta regiones de 1.8Kpb corriente arriba del sitio de inicio de transcripción. Además incluyen regiones simétricas, invertidas, repetidas y palindrómicas que podrían unir proteínas importantes en la regulación de la expresión génica. Todos los genes del complejo multigénico contienen una secuencia TATAA, la cual ha sido usada para asignar el sitio del casquete (cap). Existen además secuencias ricas en A-T (ATAAAT) a - 80 pb del sitio de iniciación de la transcripción.

E Evolución molecular.

El análisis de la secuencia de los genes ha proporcionado una información muy valiosa para reconstruír el proceso evolutivo que dió origen al actual complejo multigénico hGH-hPL. El alto grado de similitud en la secuencia de aminoácidos entre hGH, hPL y prolactina (Prl) y en las secuencias nucleotídicas de sus genes, aunado a que comparten actividades biológicas, permitió postular la hipótesis de que los miembros del complejo multigénico hGH-hPL provienen de un mismo gen ancestral común (2,10 y 11). Este ancestro común el cual se cree fué más similar a la Prl actual, dió origen mediante duplicación y divergencia a los otros miembros de la familia (12).

Se ha postulado que hace aproximadamente 350 millones de años el gen ancestral comun se duplicó dando origen a dos ramas, una para los genes hGH y hPL y la otra para el gen hPrl. Los dos genes precursores de cada rama segregaron en dos cromosomas diferentes; actualmente el gen hPrl esta localizado en el cromosoma 6 y los genes hGH y hPL en el cromosoma 17. La hipótesis postula la presencia de tres eventos de duplicación génica: el

primero originó hace ~ 400 millones de años los ancestros de la prolactina y de GH y el segundo evento dió origen a una distribución de cuatro genes: hGH-N, hPL-1, hGH-V y hPL-3. En el último evento el gen ancestral hPL-1 se duplicó hace ~5 millones de años para producir los genes hPL-4 y hPL-1. Se ha propuesto que hPL-4 se originó por duplicación del gen hPL-1 y que un mecanismo recombinacional de conversión génica permitió que el gen hPL-4 presente ahora una similitud mayor con el gen hPL-3 que con el propio gen hPL-1. La presencia de homologías internas entre estas hormonas y la abundancia de secuencias de DNA altamente repetitivas a través del locus puede explicar estos eventos recombinacionales no-usuales (13). De los 66Kpb de la secuencia nucleotídica del complejo multigénico, el 21% de las secuencias corresponden a 48 secuencias repetitivas de la familia Alu (8).

F Deleciones génicas.

El complejo multigénico hGH-hPL ha sido analizado en pacientes con niveles bajos o ausencia total de hPL durante el embarazo. En el caso de una mujer embarazada con ausencia total de hPL (14) se identificó por análisis molecular del complejo una deleción homocigótica de aproximadamente 35 kpb que incluye los genes hPL-4, hGH-V y hPL-3; otros miembros de la familia fueron heterocigotos para la deleción (15). El embarazo, parto y producto fueron normales, por lo que se pensó que el gen hPL-1 que contiene secuencias relacionadas a hPL y que no se eliminó en la deleción, podría producir una proteína que presentara actividades similares a las de hPL. En el caso de reducción parcial de hPL (16) se encontró en la madre afectada ausencia del gen hPL-4. En este caso los niveles de hPL en el suero materno fueron la cuarta parte de los valores normales. Dado que la expresión del gen hPL-3 no compensó la pérdida del otro gen hPL, esto sugiere una relación directa entre la dosis génica y la concentración de hPL en suero materno. Hasta la fecha no esta claro el porque hPL resulta dispensable durante el embarazo.

Contrariamente a lo que sucede con hPL, en un caso de deficiencia de hormona de crecimiento que se caracteriza por enanismo, se encontró al analizar el complejo multigénico una deleción de 7.5Kpb que elimina al gen hGH-N, siendo esta la causa de la ausencia de hGH (17).

II Expresión de los genes hGH y hPL.

A Clonación molecular de los DNAcs.

Trabajos realizados inicialmente en 1977 por Shine y Seeburg y col. permitieron la clonación molecular de un fragmento de 550 pb correspondiente a una porción del DNAc de hPL que hibridó al RNAm de hPL (4,18). Posteriormente Barrera-Saldaña y col. usaron esta porción del DNAc de hPL como sonda para aislar de un banco de DNAcs de placenta el DNAc de hPL completo (6). En estos estudios los RNAms obtenidos de placentas a término también fueron utilizados para construír un banco de DNAcs. El 5% de las clonas recombinantes del banco hibridaron con secuencias de DNA para hPL, indicando que el RNAm para hPL es abundante en tejido placentario a término. Estudios adicionales realizados por Barrera-Saldaña y col. detectaron dos diferentes DNAcs de hPL que fueron caracterizados mediante digestión con enzimas de restricción (18). Cuando compararon sus secuencias encontraron que existen diez diferencias en posiciones nucleotídicas, las cuales, sólo una ocasiona un cambio en la secuencia aminoacídica. Este cambio sin embargo está localizado en el péptido señalador, que se elimina al secretarse la forma madura de la hormona, por lo que se dedujo que las hormonas producidas por los dos DNAcs son idénticas.

Los DNAcs de hPL obtenidos presentan aproximadamente un 92% de similitud con la secuencia de nucleótidos del DNAc de hGH (7). Esta alta similitud entre los DNAcs de hGH y hPL condujo a realizar mapeos extensivos mediante digestión con enzimas de restricción para su caracterización. Los DNAcs de hGH y hPL pudieron ser diferenciados por la presencia de un sitio único para la enzima XbaI y la ausencia de corte con la enzima BglII en el DNAc de hPL. Por otro lado, la secuencia del DNAc de hGH presenta un sitio único para la enzima BglII y no presenta sitio de corte para la enzima XbaI (1).

B Productos a nivel de RNA.

Los transcritos primarios de los genes hGH-N, hGH-V, hPL-3 y hPL-4 tienen una longitud de 1650 nucleótidos. El procesamiento de los transcritos produce RNAms que codifican para prehormonas de 217 aminoácidos. Además, la eliminación de los intrones "splicing" por una vía alternativa del transcrito

primario del gen hGH-N produce una deleción de los primeros 45 nucleótidos del tercer exón. Este RNAm alternativo codifica para la forma variante de hGH de 20KDa (19).

Trabajos realizados por Cooke y col., demostraron que el gen hGH-V se expresa en placenta y produce también dos RNAms mediante "splicing" alternativo a nivel del cuarto intrón (20). En este estudio encontraron especies de RNAms de 900 y 1250 nucleótidos en una proporción 2:1 y esta expresión se encontró limitada al tejido de las vellosidades coriónicas de la placenta. Una de las especies de RNA se produjo por la vía de procesamiento normal que hemos descrito para hGH-N y hPL. La segunda especie de RNAm (20% del total) se originó por una vía de eliminación de intrones alternativa, en la cual, el último intrón del transcrito primario de hGH-V fué retenido, aumentando el tamaño del RNAm en 253 nucleótidos. Posteriormente, mediante histohibridación in situ, demostraron un aumento en la concentración de hGH-V durante la gestación y sublocalizaron la expresión de hGH-V en el sinciciotrofoblasto de placentas a término (21).

Entre un 10 y un 20% de los RNAms en placentas a término se encontraron específicos para hPL mientras que sólo un 0.05% fueron específicos para hGH-V. Recientemente Nickel y col. demostraron que el promotor de hPL-3 es más fuerte que el de hGH-V en células de pituitaria de rata transfectadas (22). Estos resultados indican que la expresión diferencial de estos genes esta relacionada en parte con la fuerza de sus respectivos promotores y sugiere que un mecanismo similar puede existir en placenta humana.

En el caso de los transcritos de los genes hPL activos, los tamaños de los transcritos sin procesar y procesados fueron descritos por Barrera-Saldaña y col. mediante análisis tipo Northern de los RNAs obtenidos de placenta a término (6). En este trabajo mostraron un RNAm de hPL predominante de aproximadamente 860 nucleótidos y los RNAs nucleares contenían especies de 990, 1200, 1460 y 1760 nucleótidos. Estos RNAs nucleares de alto peso molecular representan muy probablemente precursores del RNAm de hPL.

Dos sitios de iniciación de la transcripción diferentes se identificaron en los genes hPL-3 y

hPL-4 (11). La mayoría de los transcritos inician la transcripción 30 pb corriente abajo de la secuencia TATAAA y aproximadamente el 5% de los transcritos inician 30 pb corriente abajo de la secuencia CATAAA. Esta secuencia se encuentra localizada 55 pb corriente arriba de la secuencia TATAAA. Los niveles de RNAs de los genes hPL-3 y hPL-4 en placenta fueron determinados por Barrera-Saldaña y col. mediante digestión con enzimas de restricción de las clonas de DNAc de placenta, así como por análisis de transcripción inversa y digestión con nucleasa S1. Estos estudios mostraron una mayor expresión de hPL-4 en relación a hPL-3 en una proporción de 3 a 2 (18).

Por otro lado, el grupo de Chen y col. encontraron que los niveles de expresión de hPL-4 y hPL-3 constituyen el 3 y 0.5% de los RNAms de placenta, lo cual, muestra una relación de 6 a 1 en la expresión de hPL-4 y hPL-3 (8). Estudios adicionales realizados por Fritzpatrick y col., detectaron una amplia variabilidad en la relación de RNAms de hPL-4 y hPL-3 cuando analizaron los niveles de RNAms de hPL en diez placentas diferentes (23).

En relación al gen hPL-1, sus transcritos no pudieron ser detectados en placentas a término (6) y esta falta de expresión fué atribuída a la mutación que contiene este gen (G-->A) al inicio del segundo intrón (7). Interesantemente, Chen y col. en 1990 detectaron transcritos de hPL-1 en un banco de DNAc de placentas usando oligonucleótidos específicos para hPL-1. El análisis de la expresión de hPL-1 mostró que el transcrito primario puede presentar un procesamiento diferente a los transcritos de los otros cuatro genes del complejo multigénico. Debido a la transición G->A presente en el sitio donador del "splicing" del segundo intrón del gen hPL-1, en este procesamiento se utilizó un sitio donador alternativo localizado dentro del segundo intrón y un sitio aceptor localizado dentro del tercer exón. Esto último condujo a la eliminación de 24 codones de este exón. La eficiencia de esta eliminación de intrones fué muy baja; uno de cada diez transcritos entró a este vía alternativa. Por lo que el mayor porcentaje de los transcritos fueron procesados en forma incompleta con eliminación total del tercer exón (8).

C Productos a nivel de proteínas.

Los productos de expresión mejor conocidos del complejo génico son los polipéptidos hGH y hPL. hGH y hPL están constituídas por una cadena polipeptídica de 191 aminoácidos con dos puentes disulfuro y un peso molecular de 22000 daltones. Estas proteínas se sintetizan como una molécula precursora de 217 aminoácidos con 26 aminoácidos de péptido señalador. La secuencia aminoacídica de estas proteínas presenta una similitud del 85% (figura 2).

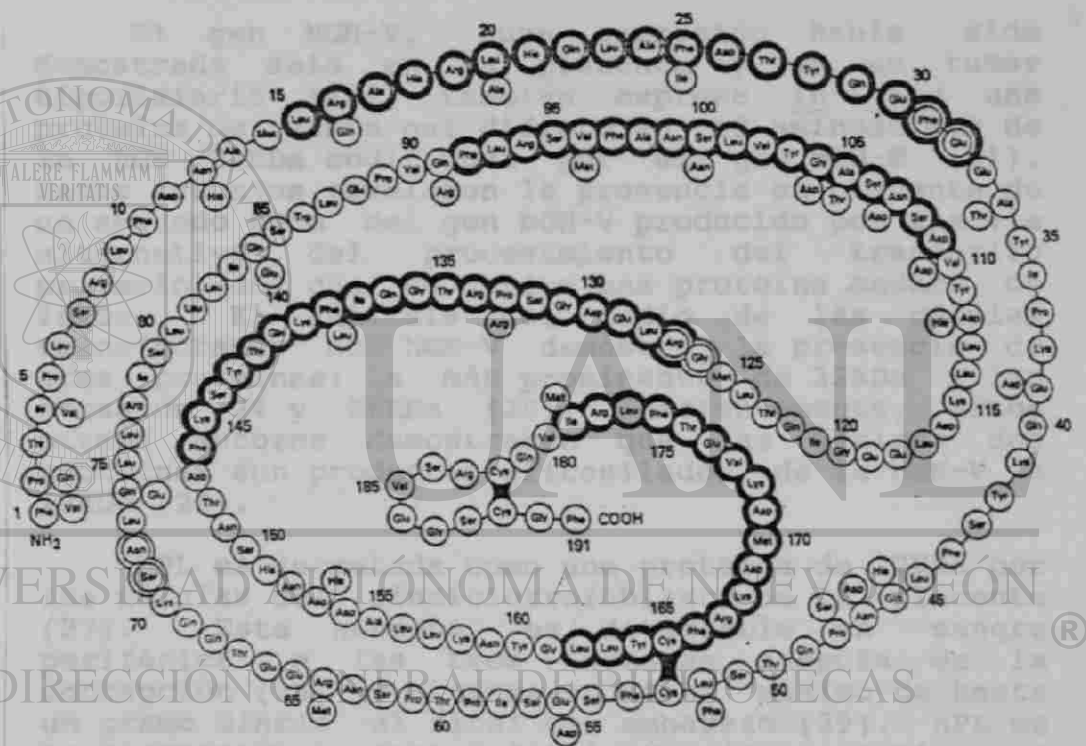

Figura 2. Comparación de las secuencias aminoacídicas de las proteínas de 22KDa codificadas por los genes hGH-N y hPL-3. El diagrama muestra la secuencia aminoacídica de hGH y las diferencias presentes en hPL-3 estan indicadas en el residuo correspondiente.

hGH constituye en la pituitaria del 1% al 3% del peso de la glándula (5-15 mg). La forma más abundante de hGH contenida en la pituitaria es la de 22KDa, aunque se han identificado otras variantes de 80, 45, 24, 20 y 17.5KDa en menor proporción (24). La variante de hGH 45KDa es un agregado o dímero originado a partir de la 22KDa. La variante 24 KDa

representa un precursor de hGH que retiene la secuencia amino-terminal de 26 aminoácidos del péptido señalador. La variante de hGH 20KDa proviene de la eliminación alternativa de intrones durante el procesamiento de las moléculas de RNAm, lo que produce una deleción de los residuos aminoacídicos 32 al 46. Esta variante constituye del 5 al 10% del total de hGH producida en la pituitaria. Por último, la hGH de 17.5KDa se origina de una deleción de 420 pb en el exón III que produce la eliminación de 40 aminoácidos en la proteína (19).

El gen hGH-V, cuya expresión había sido demostrada solo en la placenta y en un tumor hipofisiario (25), también expresa in vivo una proteína de 22KDa que difiere en 13 aminoácidos de la hGH 22KDa codificada por el gen hGH-N (21). Estos trabajos revelaron la presencia en placenta de un segundo RNAm del gen hGH-V producido por una vía alternativa del procesamiento del transcrito primario, del cual se deriva una proteína madura de 26KDa. El análisis del medio de las células transfectadas con hGH-V demostró la presencia de tres proteínas: la más prominente de 22KDa y las otras de 24 y 26KDa (20). Recientemente, estos mismos autores demostraron que las últimas dos proteínas son productos glicosilados de la hGH-V de 22KDa (26).

hPL es secretada como una proteína de 22KDa por las células del sinciciotrofoblasto de la placenta (27). Esta hormona es detectable en sangre periférica a las tres semanas después de la concepción (28) y alcanza un nivel máximo de hasta un gramo diario al final del embarazo (29). hPL se ha encontrado en sangre fetal y fluido amniótico en concentraciones de 300-1000 veces menores que en tejido normal. Por otro lado, a pesar de que transcritos del gen hPL-1 han sido detectados (8), hasta la fecha no se ha determinado si el gen hPL-1 puede producir una proteína in vivo.

Con estos antecedentes podemos resumir que mientras dos genes hGH producen al menos cuatro proteínas diferentes, la secuencias nucleotídicas de los tres genes de hPL codifican para la síntesis de una sola forma madura de hPL (ver figura 3), paradoja que requería mayor investigación al inicio del presente trabajo.

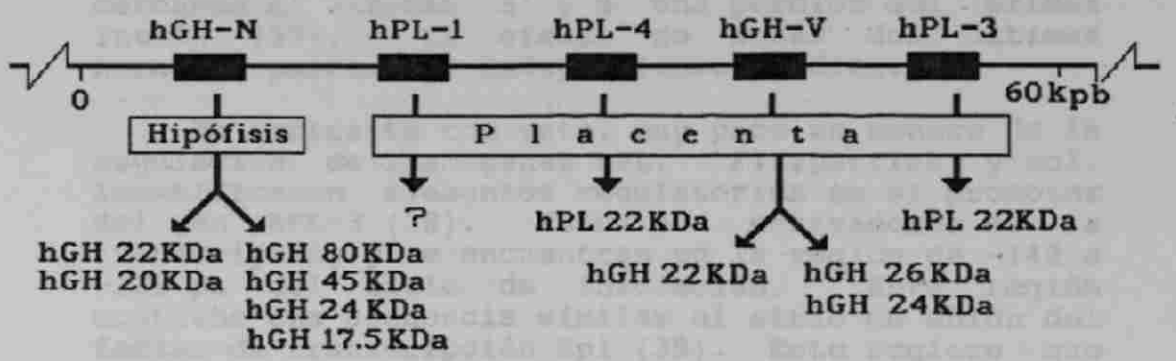

Figura 3. Expresión de los genes hGH y hPL. En este diagrama se representa la expresión específica de tejido de los genes hGH y hPL, así como sus productos a nivel de proteína.

D Regulación génica.

La alta similitud en la secuencia nucleotídica de los extremos 5' de los genes hGH y hPL (93.8%) sugiere que un mecanismo muy preciso regula su expresión específica de tejido.

El promotor de hGH contiene varias secuencias importantes para la regulación de la transcripción. Esta regulación está dada por la hormonas tiroidea (30 y 31), glucocorticoidal (29, 30) y liberadora de hormonas del crecimiento (GHRH). También se une al promotor el factor específico de pituitaria GHF-1/Pit-1 (32 y 33). Este factor es una proteína de unión al DNA que contiene un dominio "homeobox" (34 y 35) y se cree que es responsable de la expresión específica de tejido de los genes hGH y hPrl. Las secuencias de DNA requeridas para la inducción hormonal parecen ser diferentes. El receptor de la hormona tiroidea se une a la secuencia de -290 a -129 pb del sitio de iniciación de la transcripción. Estudios adicionales indican que cuatro sitios estan involucrados en la regulación de la hormona tiroidea, estos están localizados: i) 1000 pb corriente arriba del promotor, ii) dentro de las 200 pb corriente arriba del inicio de la transcripción, iii) en la secuencia TATA y iv) dentro del primer intrón del gen hGH (36). El receptor de las

hormonas glucocorticoidales se une a regiones cercanas al extremo 5' y a una porción del primer intrón (37). El efecto de estas dos últimas hormonas parece ser independiente y aditivo.

En contraste con esto, muy poco se conoce de la regulación de los genes hPL. Fitzpatrick y col. identificaron elementos regulatorios en el promotor del gen hPL-3 (38). Estos son activadores de la transcripción y se encuentran en la región de -142 a -129 pb del sitio de iniciación. Esta región contiene una secuencia similar al sitio de unión del factor de transcripción Sp1 (39). Esto sugiere que hPL-3 puede ser regulado transcripcionalmente por factores trans-actuantes como Sp1 o similares a Sp1. Estas secuencias regulatorias del promotor de hPL-3 fueron requeridas para la expresión basal pero no regularon la expresión específica de tejido.

Para explicar lo anterior, Rogers y col., en 1986 analizaron la presencia de potenciadores ó "enhancers" en el complejo multigénico hGH-hPL. Demostraron la presencia de un "enhancer" o potenciador transcripcional de 2.2 Kpb en el extremo 3' del gen hPL-3, esto es en el extremo distal del complejo multigénico hGH-hPL (40). Recientemente, Walker y col., delimitaron al "enhancer" de hPL a una región de 138 pb en el extremo 3' del gen hPL-3 (41). Este potenciador mostró ser esencial para la expresión específica de tejido. Además identificaron una secuencia de 22 pb en el "enhancer" que se une a proteínas regulatorias encontradas en células productoras de hPL. Hasta la fecha no se ha descrito ningún "enhancer" asociado al gen hGH, aunque se encontró uno en el extremo 5' del gen de la hormona de crecimiento de rata (34).

E Modelos in vitro.

El análisis de la expresión de secuencias de DNA introducidas a células en cultivo ha sido importante en estudios de regulación de la expresión génica. Los genes aislados en el laboratorio mediante técnicas de Ingeniería Genética pueden ser introducidos a células eucarióticas en cultivo.

La expresión de genes en cultivo de células puede ser transitoria o estable, dependiendo del tiempo que dure la expresión. En la primera el gen introducido generalmente por transfección permanece en forma extracromosomica y se expresa activamente

en las primeras 24-72 h, después de lo cual, es eliminado. En la segunda, se seleccionan aquellas células en las que el gen introducido se integra al genoma celular por lo que se establece una expresión continua y heredable (42).

Se ha dado gran importancia a la construcción de vectores de expresión eucarióticos que dirigen eficientemente la expresión de los genes o sus DNAcs (43). Estos vectores poseen básicamente los siguientes componentes: i) secuencias procarióticas del origen de replicación y un marcador de resistencia a antibióticos para la propagación y selección del plásmido en la célula hospedera respectivamente, ii) secuencias promotoras y potenciadores o "enhancers" que controlan la iniciación de la transcripción, eficiencia y especificidad, iii) secuencias involucradas en el procesamiento del transcrito primario y iv) el gen o DNAc que se quiere expresar.

Algunos de los vectores de expresión más eficientes se han construído utilizando señales de regulación virales. Un ejemplo de este tipo es la serie de plásmidos pSV2 que contienen el promotor del virus del simio 40 (SV40) (44). Otros vectores contienen como secuencias reguladoras las repeticiones terminales largas (LTR) del virus del sarcoma murino (VSM) ó del virus del sarcoma de Rous (45).

Se ha demostrado la síntesis, procesamiento y secreción de hGH en diferentes sistemas de cultivo celular. Pavlakis y cols., determinaron la expresión de hGH con plásmidos recombinantes que contienen los genes hGH en el vector pSV (46). Este mismo vector permitió a Lupker y col., demostrar que la prehormona es procesada correctamente por las células de mono cuando obtuvieron una línea de células Vero que expresa establemente grandes cantidades de hGH al medio extracelular (47).

La presencia de un potenciador muy potente localizado corriente arriba del gen inmediato temprano del citomegalovirus humano (hCMV) fue descrita por Boshart y col., en 1985. Durante su caracterización esta unidad promotor-potenciador mostró ser una de las unidades de control de la transcripción mas fuertes quedando evidente su utilidad para ser incorporado a vectores de expresión eucariotes (48). La potencia de la unidad

promotor-potenciador fue demostrada por Foecking y Hofstetter tanto en ensayos de expresión transitoria como estable. Esta unidad mostró ser mas potente y versátil como elemento regulatorio transcripcional que el promotor del virus SV40 y el LTR del virus del sarcoma de Rous (49). Como apoyo a los resultados anteriores Pasleu y col., demostraron una mayor expresión de bGH en células de rata GH3 con esta unidad que cuando utilizaron el LTR del virus de sarcoma de Rous (45).

Las primeras observaciones de la expresión de genes eucarióticos en cultivo celular sugirieron que la eliminación de intrones era obligatoria para la acumulación de RNAms en el citoplasma. Debido a lo anterior la llamada primera generación de vectores de expresión para DNAcs, incluyeron un intrón heterólogo además de las secuencias de poliadenilación (44). Sin embargo, en 1980 Brinster y col. usaron vectores de expresión de DNAcs de la segunda generación que no contienen intrones y demostraron que los intrones no son necesarios para la acumulación de RNAms en células transfectadas (50).

F hGH como gen de referencia.

La cuantificación de la expresión génica en sistemas de expresión transitoria está basada en determinar los niveles, ya sea de RNA o proteína, que dirige el gen transfectado. El estudio de la expresión génica en células transfectadas para lograr expresión transitoria ha sido facilitado con el uso de vectores de expresión que dirigen la síntesis de actividades enzimáticas que pueden ser determinadas fácilmente, como es el caso de la enzima cloranfenicol acetil transferasa (CAT)(51). Este ensayo de actividad de CAT ha sido utilizado ampliamente porque es fácilmente cuantificable, rápido y reproducible. El ensayo CAT ha sido tambien utilizado como gen referencia para determinar la eficiencia de los experimentos de transfección. En algunos experimentos el mismo vector de expresión contiene el gen de referencia así como el gen que se va a analizar su expresión. En otros experimentos se utilizan estos elementos en vectores diferentes, por lo que se recurre a co-transfectar las células en cultivo con los dos vectores de interés.

En 1986 Selden y col. describieron un sistema

de expresión transitoria que está basado en la detección inmunológica de la hGH secretada por las células transfectadas (52). Los niveles de hGH secretada al medio extracelular fueron proporcionales a la cantidad de RNAm citoplásmico y a la cantidad de DNA transfectado. Esta característica permitió el registro continuo de la expresión en las células transfectadas y la utilización de la expresión de hGH como gen de referencia para determinar la eficiencia de la transfección. También demostraron que el sistema de expresión transitoria de hGH en células de ratón es diez veces más sensible que el sistema de expresión CAT y es cualitativamente diferente a los sistemas generalmente utilizados porque no requiere la destrucción de las células transfectadas.

III Relaciones funcionales de hGH y hPL.

A Estructura tridimensional.

El reciente desarrollo de los métodos para purificación de proteínas y la capacidad para producir proteínas homogéneas mediante Ingeniería Genética ha hecho posible la disponibilidad de sus cristales para estudiarlos por difracción de rayos X.

hPL, hGH y la hormona del crecimiento porcino (pGH), son los tres miembros de la familia de la hormona del crecimiento que se han analizado. De estas hormonas, pGH fué utilizada por Abdel-Meguid y col. en 1987 para determinar su estuctura tridimensional (53). Esta consiste principalmente de cuatro α-hélices antiparalelas distribuídas en un paquete helicoidal enrrollado a la izquierda (ver figura 4). El alíneamiento de las secuencias aminoacídicas de pGH con otras GHs revela que los residuos dentro de las α-hélices son predominantemente invariables y por lo tanto son necesarios para mantener la integridad estructural de estas proteínas.

La alta similitud en secuencia aminoacídica de estas hormonas sugiere que la estructura porcina es básicamente la misma en las otras hormonas del crecimiento de mamíferos, por lo que se propuso como una estructura tridimensional general para todas estas proteínas (ver figura 4).

B Receptores somatogénicos y lactogénicos.

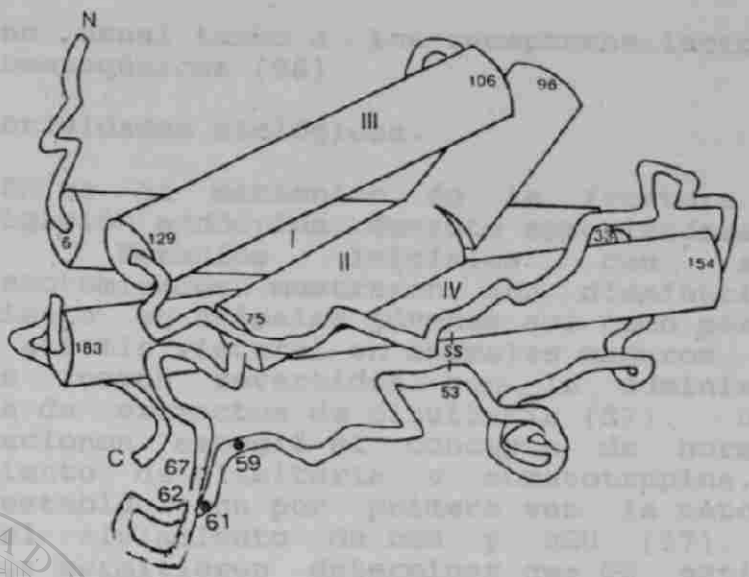

Figura 4. Modelo tridimensional de hGH basado en la estructura de la hormona del crecimiento porcino. Los cilindros representan la estructura de alfa-hélice y los residuos estan numerados de acuerdo a la secuencia de hGH.

Una gran cantidad de actividades biológicas han sido atribuídas a los miembros de la familia multigénica hGH-hPL. Estas actividades estan mediadas por la unión de las hormonas a receptores específicos en la superficie celular de las células blanco. Se han descrito dos categorías de receptores: los somatogénicos y los lactogénicos. La estructura de estos dos tipos receptores ha sido descrita (54 y 55), pero no se conoce como se lleva a cabo la transducción de la señal.

Las interacciones receptor lactogénico-ligando contribuyen al mantenimiento de la lactancia, desarrollo mamario y función reproductiva normal. Los efectos de las interacciones receptor somatogénico-ligando están relacionadas con el crecimiento lineal del hueso y con funciones metabólicas generales. Estas funciones están mediadas: i) indirectamente por la expresión del factor-1 del crecimiento parecido a insulina y ii) directamente por efecto en tejidos blanco periféricos (54).

Prl y PL se unen a receptores lactogénicos, mientras que las GHs de subprimates se unen a receptores somatogénicos. Contrario a esto, hGH y todas las GHs de primates en general se unen de una

forma no usual tanto a los receptores lactogénicos como somatogénicos (56).

C Actividades biológicas.

hGH se ha mantenido en la frontera de la investigación endócrina durante aproximadamente 100 años. Estudios iniciales con animales hipofisectomizados mostraron una disminución del crecimiento en animales jóvenes así como pérdida de peso y atrofia visceral en animales maduros. Estos efectos fueron revertidos con la administración exógena de extractos de pituitaria (57). De estas observaciones emergió el concepto de hormona de crecimiento de pituitaria o somatotropina. Li y col. establecieron por primera vez la metodología para el aislamiento de bGH y hGH (57). Estos trabajos permitieron determinar que GH estimula el crecimiento de tejido esquelético y blando y que está involucrada en el metabolismo de proteínas, carbohidratos y grasas. La función primaria de esta hormona es inducir el crecimiento proporcional de tejidos esquelético y blando. Estos efectos anabólicos son en parte mediados por factores de crecimiento dependientes de hGH llamados somatomedinas (59). La propiedad inductora de crecimiento de hGH es determinada: i) mediante la prueba de ganancia de peso, en la cual, el incremento de peso en los animales hipofisectomizados es registrado durante diez días de administración de la hormona y ii) mediante el ensayo de tibia, en el que, el crecimiento de las epífisis de las tibias en animales hipofisectomizados se mide después de inyecciones diarias de hGH. Además de la actividad somatogénica, hGH presenta efectos biológicos múltiples, como son: actividades diabetogénicas, lipolíticas, lactogénicas, similares a insulina y activación de macrófagos (60).

Por su parte, la hGH 20KDa comparte algunas actividades biológicas con la hGH 22KDa, ya que presentan la misma actividad para inducir el crecimiento y aumentar la ganancia de peso en animales hipofisectomizados (61). Sin embargo, algunas otras actividades como las similares a insulina, el efecto sobre ácidos grasos y glucosa se encuentran altamente disminuídos, pero no ausentes, en la hGH 20KDa. Por lo que esta variante, comparada con hGH 22KDa, presenta actividades marcadamente disminuídas en el metabolismo de

carbohidratos, proteínas y ácidos grasos libres (62).

Se ha postulado, que hPL regula el metabolismo materno durante el embarazo. La principal función propuesta para hPL es como un antagonista de insulina (63). hPL estimula lipólisis materna e incrementa los ac. grasos libres en la sangre materna, permitiendo su utilización como una fuente de energía. A pesar de su alta similitud con hGH, hPL no es efectiva como inductora del crecimiento ya que presenta solamente el 1% de la actividad inductora del crecimiento de hGH (64). Sin embargo, evidencia reciente sugiere que hPL regula el crecimiento y metabolismo en tejidos fetales aislados (65). Esto es apoyado por la presencia de receptores específicos para hPL en tejidos fetales humanos (66).

D Bioensayos in vitro.

La actividad biológica de hGH ha sido verificada tradicionalmente en bioensayos animales midiendo el aumento de peso corporal y el incremento de tejido esquelético blando y duro (67). Recientemente, Morikawa y col. demostraron que hGH induce la conversión adipocítica de fibroblastos 3T3-F442A de ratón e identificaron esta actividad como una propiedad específica de hGH, que no presentan otras hormonas de procedencia hipofisiaria (68). Estos mismos autores encontraron que la hGH 20KDa, también es capaz de inducir este fenómeno de diferenciación y que además este bioensayo presenta una alta sensibilidad y especificidad comparado con el bioensayo tradicional animal (69). La conversión adipocítica depende de los receptores somatogénicos en los fibroblastos 3T3, por lo que hGH actúa como un potente factor adipogénico mientras que hPL no presenta esta actividad.

Las propiedades lactogénicas de hGH fueron descritas desde hace aproximadamente 20 años y han sido confirmadas en varias especies. Uno de los bioensayos in vitro mas utilizado para medir la actividad lactogénica está basado en el efecto mitogénico de las células Nb2 de Linfoma de rata (70). En este bioensayo las células proliferan cuando se cultivan en presencia de Prl, hGH, hPL u otras hormonas lactogénicas.

E Estudios con digestiones enzimáticas.

Se han realizado múltiples estudios de digestiones enzimáticas de estas hormonas proteicas con el objeto de dilucidar las relaciones entre su estructura primaria y actividades biológicas. En estos estudios, enzimas como la plasmina, subtilisina, bromelaína, quimiotripsina, pepsina y tripsina, cortan estas moléculas en uno o más sitios, y las hormonas son convertidas en una estructura con dos o más cadenas (71). La digestión de hGH con plasmina mostró que el fragmento correspondiente a los 134 residuos del extremo amino-terminal de la proteína presentaba actividades somatotrópicas. Este fragmento pudo ser posteriormente degradado con bromuro de cianógeno y se obtuvo un fragmento de 111 aminoácidos (residuos 15-125) que presentó tanto actividad lactogénica como somatogénica (72). Posteriormente se describieron péptidos pequeños como los residuos 1-54 de hGH, ó 6-133 de bGH que presentaron una actividad significante en el aumento de las epífisis esqueléticas, por lo que la actividad biológica de hGH fué adscrita a diferentes regiones en la molécula. Por otro lado, otros estudios utilizaron fragmentos de hPL producidos mediante digestión limitada con plasmina y determinaron también que la actividad lactogénica de esta hormona se encuentra en los primeros 134 residuos aminoacídicos de la proteína (73). Resultados similares fueron obtenidos con la hidrólisis de hGH por subtilisina (74). Sin embargo en todos estos estudios de bioactividades in vitro de los fragmentos hormonales se requirieron altas dosis de fragmentos; en muy pocas ocasiones los fragmentos produjeron respuestas dependientes de la dosis, como cuando se administraron hormonas íntegras.

F Características estructurales de hGH y hPL relacionadas a actividades biológicas.

Para analizar las relaciones estructura-función en las GHs, Nicoll y col. determinaron los residuos conservados en diferentes especies (75). Las secuencias conocidas de GHs de no-primates fueron alineadas con Prls para determinar la máxima similitud con hGH y hPL. Como ya hemos descrito, hPL presenta un 85% de similitud con hGH, pero es virtualmente inactiva en el ensayo de inducción del crecimiento. De los 28 residuos en que hPL difiere de hGH; 17 fueron eliminados como residuos implicados en esta actividad porque se encuentran en

partes de la molécula que no son esenciales para la actividad de hGH y otras GHs. Además, encontraron que éstas hormonas difieren solamente en las regiones de los residuos aminoacídicos 60-68 y 99-113, cuando analizaron sus perfiles hidrofílicos e hidrofóbicos. La primer región de divergencia es causada por una sustitución de la arg 24-->met y la segunda involucra seis sustituciones, dos de las cuales causan solo ligeros cambios y las cuatro restantes causan un incremento significante en las características hidrofílicas de hPL en esta región. Estas cuatro sustituciones cambian la predicción de la estructura secundaria de la proteína, haciendo a hPL diferente de hGH y otras hGHs en esta región. Por lo tanto, estos análisis indican que las cuatro sustituciones entre los residuos 99-113 de hPL podrían ser importantes para la pérdida de su actividad inductora del crecimiento.

Por otro lado, Retegui y col. en 1982 reconocieron mediante anticuerpos monoclonales el principal determinante antigénico de hGH en los residuos 98 a 128 del extremo amino terminal de la proteina (76). Se ha descrito que el triptofano (trp) que se encuentra en la posición 86 de esta proteína no es esencial para la actividad biológica de hPL. En este estudio encontraron una actividad disminuída de hPL cuando oxidaron el residuo trp en hPL así como cuando realizaron un rompimiento de este mismo residuo. Lo anterior podría deberse a que el residuo trp está formando parte de uno de los determinantes antigénicos que permiten mantener la configuración molecular requerida para el reconocimiento por el anticuerpo específico (77). Posteriormente Neri y col. en 1984 identificaron un determinante antigénico común a hGH y hPL entre sus residuos 166 y 179 (78).

G Análisis funcional de proteínas quiméricas.

Burstein y col. y Rusell y col., en trabajos independientes, construyeron moléculas híbridas de hGH y hPL mediante la recombinación de un fragmento de hGH (residuos 1-134) con un fragmento de hPL (residuos 135-191) y viceversa. Estos experimentos fueron realizados con modificaciones químicas y/o proteólisis parcial de las proteínas y demostraron una vez más que la actividad biológica y la especificidad inmunológica de cada una de estas hormonas esta determinada por el fragmento

comprendido entre los aminoácidos 1 y 134 de estas proteínas (79, 80).

Gertler y col. en 1986 construyeron y expresaron en Escherichia colí un recombinante análogo de hGH que perdió 13 aminoácidos del extremo amino-terminal de la proteína (81). Este análogo de hGH inhibió la proliferación de las células Nb2 de linfoma y mostró una disminución en la unión a receptores somatogénicos de células Nb2 y linfocitos IM-9. Estos resultados indicaron que el extremo amino-terminal de hGH es absolutamente necesario para la unión eficiente de hGH a los receptores lactogénicos y somatogénicos.

Estudios posteriores realizados por este mismo grupo con quimeras construídas entre hGH y bGH sugirieron que el sitio de unión de hGH hacia el receptor lactogénico y somatogénico incluye el extremo amino-terminal de la molécula, mas específicamente los residuos aminoacídicos 8-18 (82, 83).

Recientemente Cooke y col. describieron la síntesis de proteínas quiméricas producto del intercambio del tercer y cuarto exón de hGH por los exones correspondientes de Prl de rata y GH de rata (84). Las proteínas quiméricas resultantes conservaron su habilidad para unirse a receptores lactogénicos pero perdieron su actividad de unión a receptores somatogénicos. Esta pérdida selectiva de unión al receptor somatogénico de las proteínas quiméricas sugiere que algunos de los determinantes estructurales de unión a receptores somatogénicos y lactogénicos son distintos y que los requerimentos estructurales del receptor lactogénico podrían ser menos estrictos que los necesarios para la unión al receptor somatogénico.

H Localización de regiones funcionales de hGH mediante mutagénesis dirigida.

Elegantes experimentos realizados por Cunningham y col. identificaron tres regiones específicas de hGH que modulan la unión al receptor somatogénico (85, 86). Estos trabajos fueron realizados mediante mutagénesis dirigida por barrido con DNA análogo y alanina y evaluaciones por unión a anticuerpos monoclonales y a receptores somatogénicos.

Las regiones que identificaron estan localizadas en: i) los residuos aminoácidos 12 al 19 codificados por el segundo exón de hGH, ii) los residuos 56 al 64 codificados por el tercer exón y iii) los aminoácidos 167 al 181 codificados por el quinto exón. Se basaron en el modelo tridimensional descrito para pGH (53), para deducir que estas tres regiones coalescen en una región discreta entre el extremo amino terminal de la hélice 1, un lazo desde la cisteína 53 al inicio de la hélice 2 y la porción central al extremo carboxi-terminal de la hélice 4 de la proteína (ver figura 5).

La región central y el extremo carboxi-terminal fueron mas importantes en la unión al receptor somatogénico que el segmento del extremo amino-terminal de la proteína. Hasta la fecha no se sabe si estas mismas regiones están involucradas en la unión al receptor lactogénico o si esta segunda especificidad es distinta estructuralmente.

En una serie de estudios realizados por Retegui y col. encontraron 20 determinantes antigénicos en la superficie de hGH. Estos trabajos demostraron que los dominios antigénicos de hGH estan localizados en la porción central de la secuencia. Estos son los residuos aminoacídicos 1 al 32 codificados por el segundo exón. Esta porción interactúa parcialmente con los residuos 106-128 codificados por el cuarto exón y con los residuos 155-191 codificados por el quinto exón de hGH (87, 88).

Por otro lado, el grupo de Vogel y col. construyó y expresó en Escherichia coli dos nuevos análogos de hGH para determinar las propiedades de unión a los receptores somatogénicos y lactogénicos (89). Uno de los análogos de hGH construídos contenía una deleción de siete aminoácidos en el extremo amino-terminal de la proteína, además de dos mutaciones puntuales en las cuales la arg en la posición 8 fué sustituída por metionina (met) y el aspártico (asp) en el sitio 11 por alanina (ala). El otro análogo además de contener los primeros 13 aminoácidos de bGH ya descritos por estos mismos autores, contenía una elongación de los aminoácidos met y ala en el extremo amino-terminal y una mutación puntual que condujo a la sustitución del residuo ala en la posición 11 por asp. Estos trabajos indican que los dominios de hGH involucrados en la unión a los receptores

somatogénicos y lactogénicos no son idénticos. Además, sus resultados sugieren que el asp en el sitio 11 encontrada en los GHs de primates y Prls (no encontrada en GHs de no-primates) no es importante para la actividad lactogénica en el bioensayo de células Nb2. Por otro lado, determinaron que los residuos aminoacídicos del 8 al 13 son definitivamente esenciales para la actividad de hGH, pero se propone que afectan indirectamente su actividad, estabilizando la siguiente parte de la α-hélice, por lo que la eliminación de esta región podría afectar la estructura secundaria de la proteína.

Recientemente, el grupo de Uchida y col. utilizó también mutagénesis dirigida para construír cuatro mutantes artificiales de hGH (90). Dirigieron las modificaciones a la región central del lazo localizada en los aminoácidos 54 a 74 previamente descrita por el grupo de la compañía Genentech (86). Los cambios nucleotídicos condujeron al reemplazo de la prolina en la posición 59 ó 61 por alanina y a la deleción de los residuos aminoacídicos 62 al 67 de hGH. Las proteínas mutantes purificadas mostraron que los residuos 62 al 67 deletados (ver figura 4) son determinantes importantes para la diferenciación de células preadipocíticas 3T3-F442A hacia adipocitos. Por lo que se sugiere que esta región podría ser un requerimiento común para la unión al receptor somatogénico de hGH.

HIPOTESIS

Las secuencias nucleotídicas de dos de los tres genes hPL codifican una sola forma madura de la hormona y se desconoce si el tercero es funcional. Como se ha detectado gran variabilidad en la expresión de estos genes y aun no se ha demostrado su contribución a la producción placentaria de hPL, si analizamos la expresión de cada uno de ellos en un modelo in vitro, determinaremos su potencial de codificación y podremos identificar secuencias nucleotídicas responsables de sus diferencias en niveles de expresión.

OBJETIVOS

1. Desarrollar un sistema eficiente para detectar productos de expresión derivados de genes introducidos a células en cultivo.

2. Determinar la expresión y el potencial de codificación in vitro de los genes hPL.

3. Verificar la actividad biológica de las hormonas producidas en cultivo celular en el modelo de diferenciación adipocítica.

MATERIALES Y METODOS

I Enzimas, estuches comerciales, radioisótopos, bacterias y plásmidos.

Obtuvimos las enzimas de restricción y modificación de las compañías Bethesda Research Laboratories (Gaithersburg, MD E.U.A.) y New England Biolabs, (Berverly, MA E.U.A.) y para su utilización seguimos las recomendaciones de los fabricantes. Para la cuantificación de hGH por radioinmunoensayo (RIA) utilizamos un estuche comercial de Diagnostic Products Co. (Los Angeles, CA E.U.A.). Los nucleótidos radiactivos [^{32}P]-αdCTP y [^{35}S]-metionina los adquirimos de Amersham International (Buckinghamshire, Inglaterra). Las cepas de Escherichia coli utilizadas fueron HB101, RR1 y JM101. Aunque los plásmidos utilizados no ofrecen riesgo alguno para la salud, trabajamos siguiendo las recomendaciones internacionales de los Institutos Nacionales de la Salud en E.U.A. El plásmido pNUT (91) fué obsequiado por el Dr. Richard Palmiter de la Universidad de Washington en Seattle, E.U.A. El gen hGH-N cuyo aislamiento ya ha sido descrito a partir de un banco de genes humanos (92), fué donado a nuestros laboratorios por el Dr. Grady Saunders de la Universidad de Texas en Houston, E.U.A., mientras que el plásmido pCMVCat fué un obsequio del Dr. Hans Hofstetter de la compañía CIBA-GEIGY. El DNAc de hPL, fragmento utilizado como rastreador molecular, fué derivado del plásmido phPL815 (6). Dicho fragmento presenta un 90% de similitud en secuencia con el DNAc de hGH.

II Construcción de moléculas recombinantes.

Los fragmentos de DNA requeridos para los experimentos de clonación molecular fueron purificados a partir de geles preparativos de agarosa o poliacrilamida. Esto lo efectuamos ya sea por electroelución o por la técnica de adsorción a vidrio (93).

Realizamos las reacciones de ligación a 16°C por 18 h en volúmenes de 10 a 20 µl. Empleamos una relación molar de 3 a 1 del DNA del inserto con respecto al vector. Precipitamos el material ligado con etanol y resuspendimos en TE (10 mM Tris-HCl y 0.1 mM EDTA, pH 7.5). Empleamos este material para transformar (94) las cepas HB101 o RR1 de Escherichia coli. Aislamos los plásmidos recombinantes a partir de 3 ml cultivo y los sometimos a un análisis rápido con

enzimas de restricción y finalmente preparamos el DNA plasmídico a gran escala (94). Caracterizamos los plásmidos recombinantes en detalle mediante digestiones con enzimas de restricción diagnósticas, electroforésis en geles de agarosa y poliacrilamida y análisis de DNA tipo Southern (95). Finalmente obtuvimos tres preparaciones independientes de los DNAs plasmídicos mediante la técnica de preparación a gran escala (94) para asegurar una calidad uniforme de las preparaciones y evitar variaciones en los experimentos posteriores donde se introducirían estos a células en cultivo.

A partir del plásmido pMThGH, recuperamos con la ayuda de cortes con enzimas de restricción un fragmento de 2.1 Kpb que contiene, en la estructura del gen, la región que va desde el sitio BamHI en el nucleótido +2 (es decir inmediato al inicio del RNAm) hasta el sitio EcoRI localizado aproximadamente 600 nucleótidos más allá de la señal de poliadenilación. Como siguiente paso de la estrategia insertamos este fragmento de BamHI a EcoRI entre los mismos sitios de la región de sitios múltiples de clonación (SMC) del vector de expresión pAVE1, dando lugar a pAVE1hGH.

Los genes hPL sin sus promotores fueron insertados en pNUT, al cual se le habían eliminado previamente las secuencias del gen hGH. Las construcciones se llevaron a cabo al ligar el fragmento mayor BamHI-EcoRI de pNUT, con uno o varios fragmentos de DNA conteniendo los genes de interés. Las regiones génicas hPL subclonadas en pNUT, consistieron de las secuencias de estos genes desde el sitio natural BamHI (excepto por hPL-1), ubicado entre el segundo y sexto nucleótido del primer exón, hasta el sitio EcoRI natural o artificial (creado en el extremo 3' del gen hPL-1, con la ayuda de enlazadores moleculares) localizado varios cientos de pares de bases mas allá del sitio de poliadenilación de los genes.

Construímos plásmidos recombinante híbridos entre los genes hGH-N, hPL-3 y hPL-4. Realizamos una digestión con las enzimas de restricción BamHI y EcoRI de los plásmidos pNUT, pNUThPL-3 y pNUThPL-4, recuperamos los fragmentos correspondientes a los genes hGH-N y hPL-3 y el vector pNUT(-). Posteriormente digerimos los genes hGH, hPL-3 y hPL-4, con la enzima SacI y obtuvimos dos fragmentos de cada gen. Los fragmentos más pequeños contienen el primero y segundo exones de los genes y los más grandes contienen los últimos tres exones de los genes. Ya que obtuvimos los fragmentos realizamos las siguientes ligaciones:

vector pNUT de 5247pb con el fragmento BamHI-SacI de hPL-3 de 582pb que contiene los dos primeros exones y con el fragmento SacI-EcoRI que contiene los últimos tres exones de hGH. El producto de esta ligación fue denominado: GH(PL-3:I,II).

vector pNUT con el fragmento BamHI-SacI de hPL-4 y el fragmento SacI-EcoRI de hGH. El plásmido recombinante fue llamado: GH(PL-4:I,II).

vector pNUT con el fragmento BamHI-SacI de hGH y el fragmento SacI-EcoRI de hPL-4. El plásmido recombinante obtenido fué denominado: PL-4(GH:I,II).

III Cultivo celular, expresión transitoria y marcaje de proteínas secretadas.

Las células COS-7 de riñón de mono donadas por el Dr. Tien Kuo de la Universidad de Texas en Houston, E.U.A. fueron adaptadas a crecer en medio Eagle modificado por Dulbecco (Sigma Chemical Co., St. Louis MO) conteniendo 5% y 1% de suero fetal de ternera (SFT, HyClone Laboratories, Inc., Logan UT E.U.A.). El mantenimiento y propagación de las células se realizó a 37°C en atmósfera húmeda conteniendo 5% de CO_2.

Expusimos 3 X 10^6 células COS-7 a 7.5 ug de DNA plasmídico preparado como precipitado fino por la técnica de fosfato de calcio (96). Evaluamos la eficiencia de transfección en cada experimento usando el ensayo (97) de cloranfenicol acetil transferasa (CAT). Este ensayo lo practicamos en extractos de células cotransfectadas con los plásmidos de interés y el plásmido pCMVCat (98).

Para marcar las proteínas sintetizadas de novo transfectamos las células COS-7 y 48 h después sustituímos el medio de cultivo inicial por medio libre de metionina conteniendo 1% de SFT dializado y metionina marcada con [^{35}S] (Amersham Intl., Buckinghamshire, Inglaterra). Agregamos 12.5 ó 25 microcuries (μCi) de [^{35}S]-metionina por ml de medio de cultivo y continuamos la incubación por 4 h más. Para el análisis electroforético de proteínas marcadas radiactivamente precipitamos las proteínas del medio con cuatro volúmenes de acetona fría, determinamos su concentración por el método de Bradford (99) y las resuspendimos en amortiguador para electroforesis (100). Desnaturalizamos las proteínas, por ebullición durante 2 min en presencia de SDS y ß-mercaptoetanol y enseguida las sometimos a electroforesis en geles

discontinuos de poliacrilamida-SDS de 5% y 13%. Colocamos los geles sobre papel filtro Whatman 3MM y secamos al vacío por 1 h a 60°C. Una vez secos, los expusimos a películas ultrasensibles de rayos X por tiempos variables a temperatura ambiente (autorradiografía).

Las cuantificaciones de hGH se realizaron mediante RIA empleando el estuche comercial anteriormente descrito. Medios de cultivo o extractos celulares fueron diluidos y procesados, usando el protocolo incluído con el estuche, con las modificaciones brevemente descritas a continuación: se disminuyó el volúmen de reacción a la mitad y se aumentó el tiempo de incubación de 60 a 90 min en la primera fase de incubación y de 60 a 75 min en la segunda.

IV Aislamiento de RNA, hibridación en línea "slot dot" e *hibridación tipo "Northern"*.

Recuperamos los RNA totales de las células transfectadas por el método de isotiocianato de guanidina-fenol-cloroformo (101). Colectamos las células 48 h después de la transfección y aislamos los RNAs totales por el método mencionado anteriormente. Posteriormente desnaturalizamos los RNAs y los separamos en un gel agarosa-urea-ácido al 5% para analizar su integridad.

Cuando realizamos estudios de hibridación en línea "slot dot" depositamos por duplicado 3 µg de los diferentes RNAs desnaturalizados en una membrana de nitrocelulosa previamente colocada en el ensamble del sistema "vacusystem". Horneamos las dos tiras de nitrocelulosa con los diferentes RNAs e hibridamos estas con la sonda previamente marcada radiactivamente por el método de oligonucleótidos al azar (102). Utilizamos como sondas o rastreadores moleculares el DNAc de hPL y el DNAc de DHFR obtenido del plásmido pNUT. Este último fue utilizado como un reportero interno para estandarizar las eficiencias de transfección en los diferentes experimentos. Además, para validar los resultados tomando en consideración variaciones en la eficiencia de transfección, cuantificamos la cantidad de sonda DHFR hibridada y los valores obtenidos los usamos para normalizar las cantidades de RNAs depositados en un nuevo experimento.

Para el análisis de RNA tipo "Northern" desnaturalizamos los RNAs mediante glioxilación, los separamos por electroforésis en geles de agarosa de

acuerdo a su tamaño (103) y los transferimos por capilaridad a membranas de nitrocelulosa (104). Las prehibridaciones, hibridaciones, lavados y autorradiografías de los filtros las realizamos usando técnicas estándares (95).

V *Bioensayo de hGH en el modelo de diferenciación adipocítica.*

Se cultivaron células 3T3-F442A como preadipocitos en medio esencial mínimo de Eagle (MEM) modificado por Dulbecco y suplementado con 5 μg/ml de insulina, 10^{-6}M de biotina y una mezcla de 5% de suero de gato con 0.5% de suero de ternera. Para el ensayo se disgregaron las células en crecimiento exponencial con una solución de tripsina al 0.1% e inocularon en cajas de 35 mm de diámetro. Las células se cultivaron en el medio no adipogénico anteriormente descrito y cuando estuvieron confluentes reemplazamos éste por el medio definitivo de conversión (105).

La actividad adipogénica de las proteínas quimeras se analizó cuando agregamos, al medio no adipogénico de fibroblastos 3T3, un 30% del medio de las células COS-7 transfectadas con los plásmidos recombinantes. Este último contenía las proteínas quimeras producidas por las células COS-7 transfectadas con los plásmidos recombinantes híbridos. Utilizamos como testigo negativo el medio de las células que no secretaron hGH porque fueron transfectadas con el vector pNUT(-), el cual carece de secuencias codificantes para la hormona y como testigo positivo el medio de las células transfectadas con el plásmido pNUT que contiene secuencias para hGH. 7 días después de agregarles el medio definitivo de conversión, las células fueron fijadas y teñidas con el colorante rojo oleoso O para determinar la acumulación de lípidos. Posteriormente cuantificamos el número de agrupamientos de células adiposas y determinamos el porciento (%) de conversión adipocítica (figura 5A).

Por otro lado, también introdujimos los plásmidos recombinantes en los fibroblastos 3T3 (ver figura 5B) por la técnica de co-precipitación con fosfato de calcio-DNA (96). Posteriormente 7 días después determinamos el % de conversión adipocítica como ya describimos en el párrafo anterior.

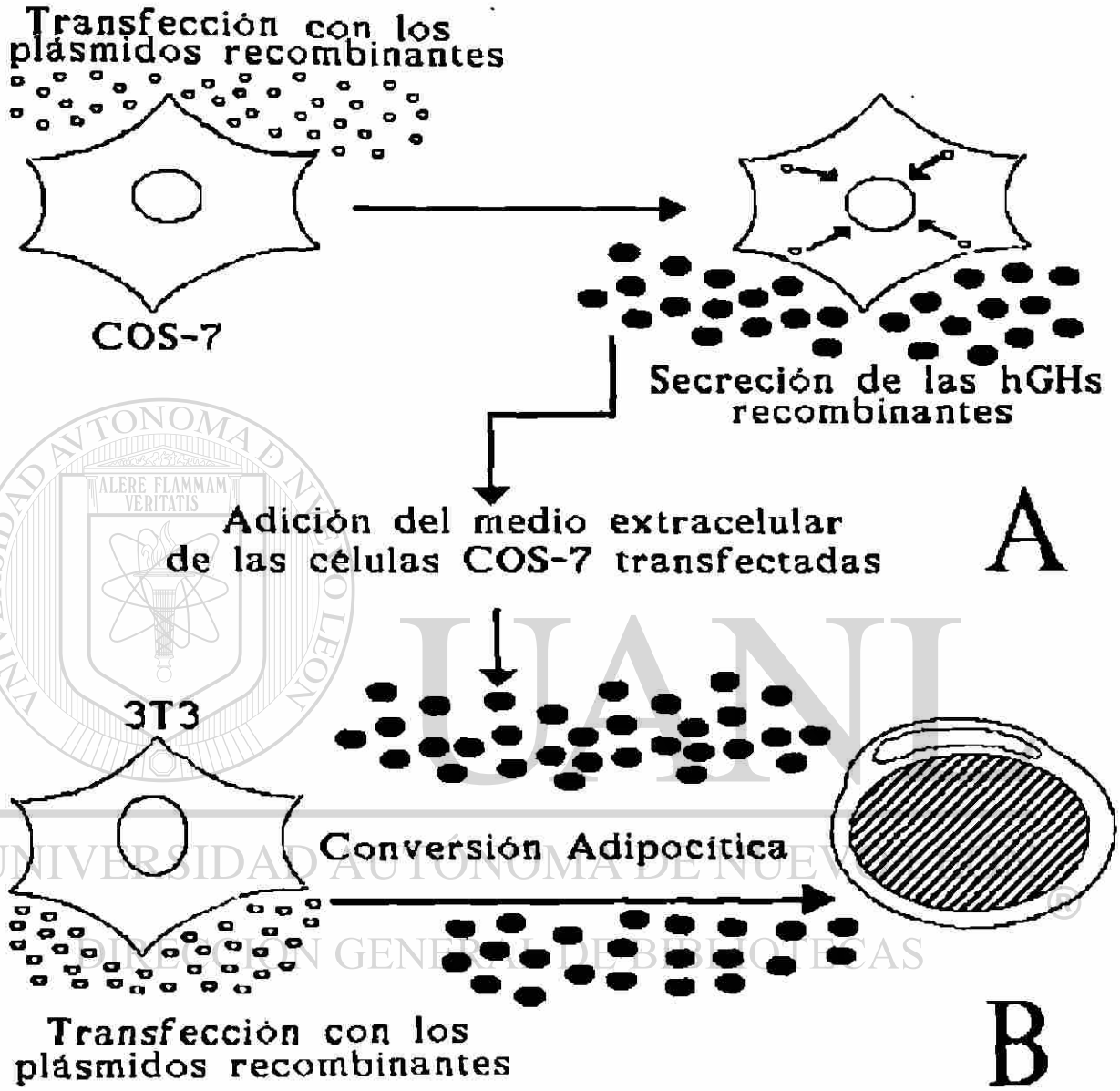

Figura 5. Bioensayo de hGH en el modelo de conversión adipocítica. En el diagrama se muestra la conversión adipocítica de las hGHs recombinantes producidas por las células COS-7 y adicionadas a cultivos de preadipocitos 3T3 (A) y de las hGHs producidas directamente por las células preadipocíticas transfectadas con los plásmidos recombinantes (B).

RESULTADOS

I. Adaptación de un modelo *in vitro* para la expresión y análisis funcional de hGH.

A Construcción de un vector para la expresión de hGH en cultivo celular.

Iniciamos nuestro trabajo con el vector de expresión pAVE1 previamente construído en nuestro laboratorio por Ramiro Ramírez Solís (106). Este vector posee una gran versatilidad para insertar y colocar regiones estructurales de genes bajo el control transcripcional de la potente unidad promotor-potenciador del hCMV. Decidimos probar la habilidad de este vector para expresar genes de mamíferos, optamos por usar la región estructural del gen cromosomico hGH-N (conteniendo aún los intrones), el cual ya ha sido probado y propuesto como un excelente gen de referencia para estudios de expresión transitoria de genes (50). Procedimos como se ilustra en la figura 6. En esta figura se muestra que a partir del plásmido pMThGH, recuperamos con la ayuda de cortes con las enzimas de restricción BamHI y EcoRI un fragmento de 2.1 Kpb que comprende, en la estructura del gen, la región que va desde el nucleótido +2 (es decir inmediato al inicio del RNAm) hasta aproximadamente 600 nucleótidos más allá de la señal de poliadenilación. Como siguiente paso de la estrategia, insertamos este fragmento BamHI-EcoRI entre los mismos sitios de la región de SMC de pAVE1, dando lugar a pAVE1hGH.

Una vez que obtuvimos el plásmido recombinante pAVE1hGH; caracterizamos éste con enzimas de restricción diagnósticas y realizamos la preparación de su DNA a gran escala. Obtuvimos el DNA de buena calidad y en cantidad suficiente para los experimentos posteriores con células en cultivo.

El DNA del vector pAVE1hGH fué introducido transitoriamente a células COS-7 en cultivo mediante el método de co-precipitación con fosfato de calcio. 48 h más tarde cuantificamos mediante RIA la producción de hGH por las células transfectadas. Este análisis fué practicado tanto en el medio intracelular como en el extracelular de las células transfectadas. Encontramos en el medio extracelular de las células transfectadas 1.2µg de hGH/caja de cultivo de 75 cm^2 lo que correspondió a un 98% del

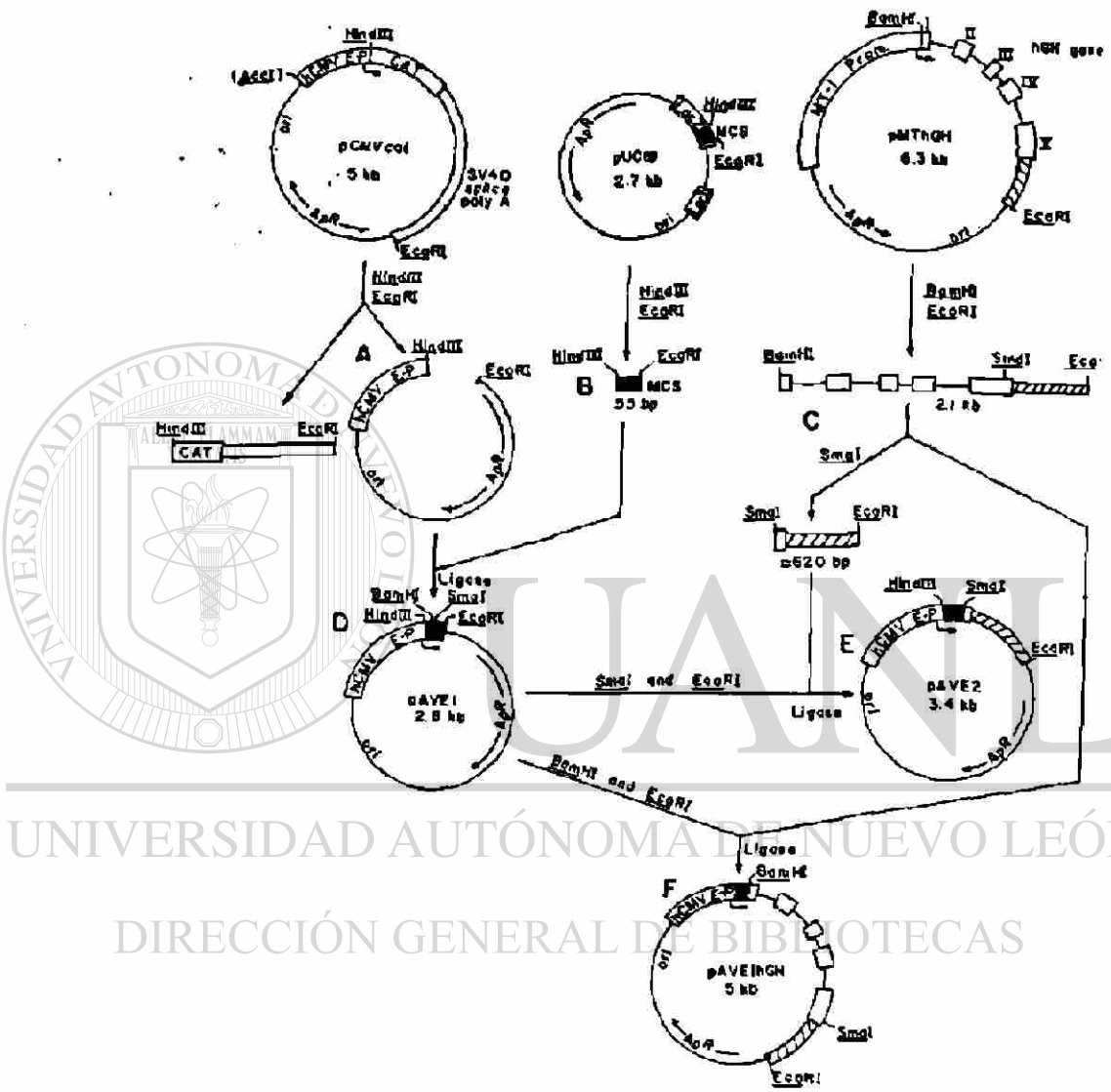

Figura 6. Estrategia para la construcción del vector de expresión pAVE1hGH. Los fragmentos aislados a partir de los plásmidos pMThGH (C) y pAVE1 (D) fueron ensamblados en (F) para dar lugar a pAVE1hGH. Kilopares de bases esta representada como Kb.

total de hGH producida por las células COS-7 transfectadas.

B Selección del vector de expresión mas eficiente.

Para asegurarnos de emplear el vector de expresión más eficiente a nuestro alcance para la expresión de genes eucarióticos en cultivo, comparamos la eficiencia de expresión de hGH del vector pAVE en relación con otros vectores que poseen elementos de control transcripcionales y que son comunmente utilizados.

Transfectamos células COS-7 con los DNAs de las construcciones quiméricas de interés: pSVgpthGH, pMThGH, pNUT y pAVE1hGH (ver figura 7). Estos vectores contienen los siguientes elementos de regulación: en el caso del pSVgpthGH, el gen hGH se encuentra bajo el control de su propio promotor natural (P) y de una copia de potenciador o "enhancer" del virus SV40. En pMThGH, el gen estructural hGH se encuentra fusionado al promotor de la metalotioneína de rata (MT). pNUT contiene el promotor de MT así como el potenciador de SV40 para expresar hGH. Finalmente pAVE1hGH, como ya describimos anteriormente, contiene el promotor-potenciador de hCMV dirigiendo la expresión de hGH.

Cuantificamos mediante RIA la cantidad de hGH secretada al medio de cultivo por las células transfectadas con cada uno de estos plásmidos recombinantes. En estos experimentos detectamos por RIA los niveles más altos de expresión en las células COS-7 transfectadas con los plásmidos pNUT y pAVE1hGH (ver figura 8). Por lo que seleccionamos estos dos vectores de expresión para los estudios subsecuentes.

C Expresión de los DNAcs de hGH en cultivo celular.

Por otro lado, confirmamos la selección anterior cuando analizamos la expresión de los DNAcs de hGH. Los DNAcs de las hGHs de 20 y 22KDa fueron obtenidos en nuestro laboratorio mediante clonación preferencial por Diego E. Rincón Limas (107). Estos minigenes fueron insertados en el vector de expresión pAVE2 (108) por Luis E. Alvídrez Quihui (109).

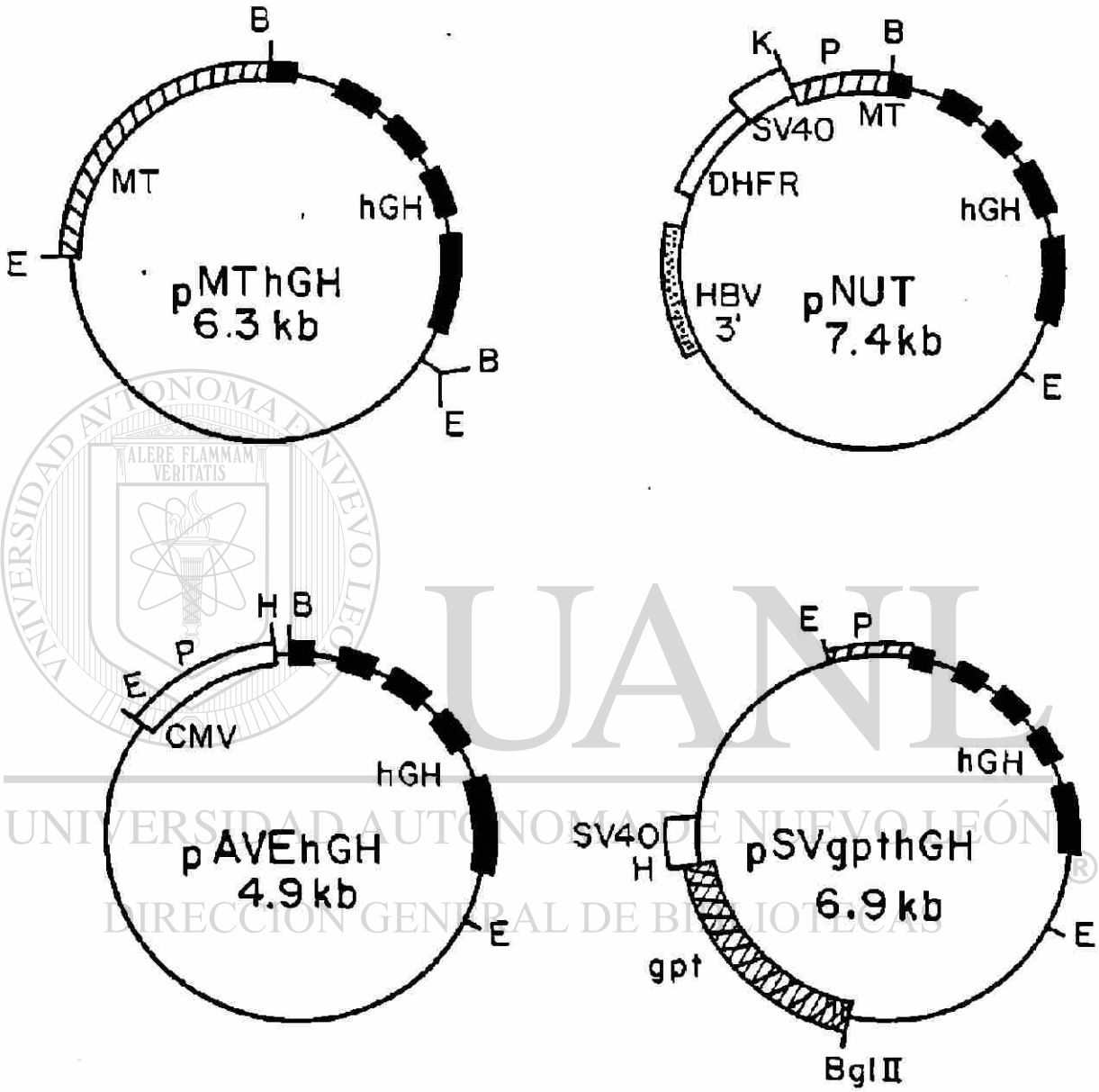

Figura 7. Estructura de los vectores de expresión de hGH. Todos los vectores de expresión, con excepción de pSVgpthGH contienen el gen hGH sin su promotor natural fusionado a los diferentes elementos de control transcripcional indicados.

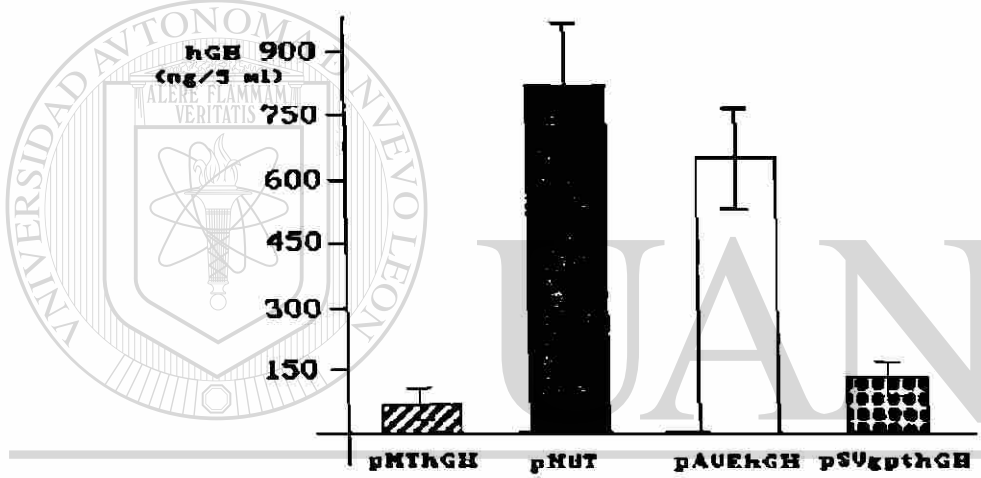

Figura 8. Expresión transitoria de hGH en células COS-7 transfectadas con los diferentes vectores de expresión. Introdujimos los plásmidos recombinantes en células COS-7 mediante la técnica de co-precipitación de fosfato de calcio-DNA. 48 h después cuantificamos mediante radioinmunoensayo la secreción de hGH al medio de cultivo. Los valores de hGH corresponden al promedio y desviación estandard de tres experimentos independientes.

Para analizar la expresión de estos DNAcs procedimos a introducirlos a las células COS-7 en cultivo. Utilizamos los plásmidos que contenían los nuevos minigenes hGH 22KDa (pAVEhGH22K) y hGH 20KDa (pAVEhGH20K), así como el DNA de plásmidos testigos pAVE1 y pAVE1hGH. 48 después recuperamos los medios de cultivo de las células COS-7 transfectadas con los diferentes vectores de expresión. Los valores de RIA para hGH detectados en estos experimentos se muestran en la tabla I; los valores de hGH secretados por las células transfectadas con pAVEhGH22K promediaron 1259 ng por frasco de cultivo de 75 cm^2 de superficie. En el caso de pAVEhGH20K estos promediaron 41 ng. Esta marcada diferencia en valores de hGH determinados mediante RIA no la esperábamos y después de confirmar su reproducibilidad, nos propusimos encontrarle explicación.

Decidimos entonces realizar la detección de las proteínas hGH22KDa y hGH20KDa expresadas y secretadas al medio en los experimentos de transfección.

Tabla I. Expresión transitoria de hGH en células COS-7 transfectadas.

Plásmido transfectado	Producción de hGH * $\bar{X} \pm$ D.S.
pAVE 2	2.3 ± 2.0
pAVE1hGH	1252.0 ± 169.0
pAVEhGH22K	1258.8 ± 158.3
pAVEhGH20K	41.1 ± 23.4

* Los valores son en ng de hGH por frasco de cultivo de 75 cm^2 obtenidos mediante RIA en alícuotas de los medios y corresponden al promedio ($\bar{X}$) y desviación estandar (D.S.) de tres experimentos independientes.

D Detección de las hGHs secretadas por las células COS-7.

Primero realizamos los experimentos para detectar las proteínas sintetizadas de novo por las

células transfectadas. Para ello, marcamos con [^{35}S]-metionina las proteínas sintetizadas de novo por las células transfectadas con el plásmido pNUT. En la figura 9 se muestra el autorradiograma de un experimento de expresión transitoria donde utilizamos concentraciones de 25µCi y 50µCi de metionina marcada con ^{35}S. Una banda nueva, ausente en el medio de las células transfectadas con el vector solo [carril (-)] apareció en el medio de las células transfectadas (carriles marcados con 25µCi y 50µCi). Esta nueva banda migró en la posición esperada para la hGH purificada de hipófisis humanas. Además, detectamos una segunda banda de menor intensidad y tamaño (20KDa), la cual también comigró con la forma variante de hGH menos abundante en la hipófisis. Estos resultados cumplieron nuestro objetivo inicial de visualizar proteínas secretadas derivadas de genes transfectados mediante marcaje con 25 y 50 µCi de [^{35}S]-metionina. Como en ambos casos obtuvimos buenos resultados utilizamos la concentración menor.

Una vez que implementamos los experimentos para visualizar las proteínas sintetizadas de novo, evaluamos la expresión de las hGHs del 22KDa y 20KDa. Para ello, realizamos transfecciones con los minigenes hGH 20KDa y 22KDa y llevamos a cabo el marcaje de proteínas con [^{35}S]-metionina. Posteriormente analizamos las proteínas secretadas por las células COS-7 transfectadas mediante electroforesis en gel de poliacrilamida-SDS y autorradiografía. La figura 10 muestra tanto la proteína hGH22KDa como la hGH20KDa con señales autorradiográficas específicas y con diferencias en abundancia mucho menores que las detectadas por el RIA. Los resultados anteriores confirmaron nuestra sospecha referente a la ineficiente detección inmune de la variante de hGH 20KDa por el anticuerpo específico para hGH 22KDa.

E Actividad adipogénica de las hGHs recombinantes producidas in vitro.

Ademas, verificamos, la actividad funcional de las hGHs recombinantes producidas in vitro en el modelo de diferenciación de fibroblastos preadipocíticos de la línea celular 3T3-F442A hacia adipocitos (105).

Cuando agregamos alícuotas del medio de las células transfectadas con pAVE1hGH al medio de

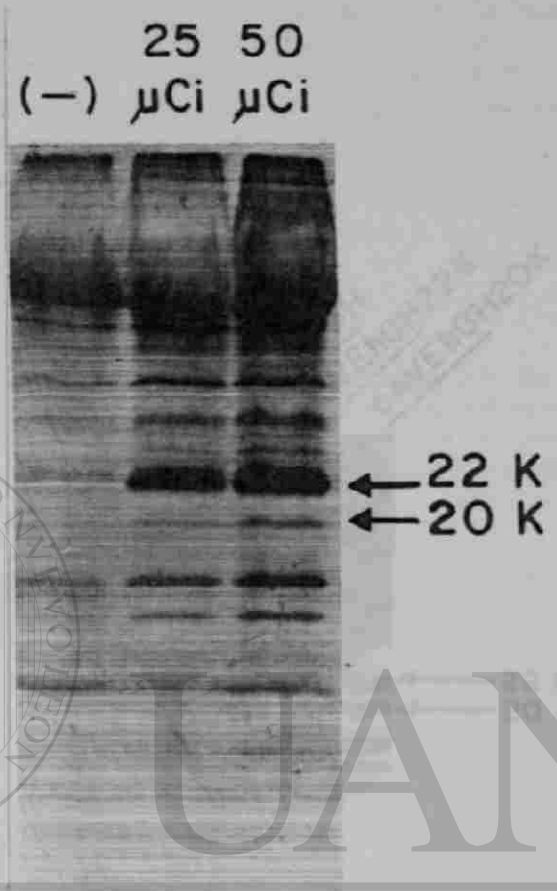

Figura 9. Detección de proteínas secretadas por las células transfectadas. Analizamos los medios de cultivo de las células COS-7 transfectadas mediante electroforesis en gel de poliacrilamida-SDS y autorradiografía para determinar la presencia de proteínas radiactivas secretadas. Las proteínas sintetizadas de novo fueron marcadas radiactivamente usando 25 ó 50µCi de [^{35}S]-metionina. La autorradiografía muestra la presencia de dos nuevas bandas (flechas) presentes en el medio de cultivo de las células COS-7 a las que les introdujimos el plásmido pNUT (carriles marcados 25 y 50µCi). El testigo negativo [(-)] correspondió al medio de las células transfectadas con el derivado del plásmido pNUT al que se le eliminó la región codificante para hGH. El tamaño de las proteínas hGH se indica en KDa (K).

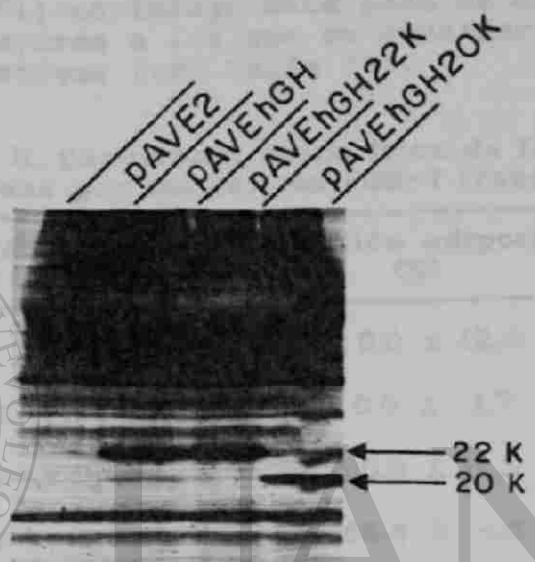

Figura 10. Detección de las hGHs de 20 y 22KDa en el medio de las células COS-7 transfectadas con los plásmidos recombinantes. Realizamos un marcaje con [^{35}S]-metionina de las proteínas secretadas por las células COS-7 transfectadas. La autorradiografía muestra la presencia de dos nuevas bandas (flechas) presentes en el medio de las células transfectadas con el vector pAVE2, conteniendo ya sea el gen (pAVEhGH), el DNAc de hGH 22KDa (pAVEhGH22K) o el DNAc de hGH 20KDa (pAVEhGH20K). Estas nuevas proteínas se encuentran ausentes en el medio de las células a las que se les introdujo el vector solo (pAVE2). El tamaño de las proteínas hGH se indica en valores de KDa enseguida de las flechas (K).

cultivo de los preadipocitos, una fracción significativa (65%) de ellos, se diferenciaron hacia adipocitos. El medio de las células COS-7 conteniendo la hGH 22KDa mostró un 100% de conversión adipocítica. Además, células 3T3 suplementadas con el medio conteniendo la hGH 20KDa alcanzaron un 66.4% de conversión adipocítica. El medio de las células transfectadas unicamente con el vector (pAVE1) no indujo este paso de diferenciación a niveles mayores a los que se consideran de fondo o no significativos (ver tabla II).

Tabla II. Conversión adipocítica de las hGHs secretadas por las células COS-7 transfectadas

Plásmido transfectado	Conversión adipocitica * (%)
-	0.0 ± 12.6
pAVE1	0.0 ± 1.7
pAVEhGH22K	100.0 ± 11.5
pAVEhGH20K	66.4 ± 4.5

* Los valores corresponden al promedio y desviación estandard de tres experimentos independientes.

Las evidencias anteriores demuestran que las hGHs de 22KDa y 20KDa secretadas por las células COS-7 fueron capaces de inducir la conversión adipocítica de los fibroblastos preadipocíticos 3T3, aunque a niveles diferentes.

Los resultados que obtuvimos en esta subsección fueron utilizados para la publicación del artículo: New vectors for the efficient expression of mammalian genes and their complementary DNAs in cultured cells. Además, algunos de estos resultados fueron incluídos en la revisión: The human placental lactogen and growth hormone multigene family y en el artículo que fué sometido a publicación en Biochemical Biophysical Acta: Functional expression of human pituitary growth hormone complementary DNAs (ver anexo A).

I Potencial de codificación *in vitro* de los genes hPL.

Una vez que desarrollamos el modelo experimental que nos permitió cuantificar la expresión funcional de hGH *in vitro*, lo utilizamos para determinar la expresión de los genes hPL.

A Construcción de una nueva serie de vectores para la expresión de los genes hPL.

Las secuencias estructurales de todos los genes hPL sin sus promotores naturales fueron insertados en el vector de expresión pNUT previamente seleccionado en la sección anterior. Para realizar esto, reemplazamos las secuencias del gen hGH-N en pNUT, por aquellas correspondientes a los genes hPL. Todos los genes de la familia hGH-hPL excepto hPL-1 poseen un sitio BamHI único entre el segundo y séptimo nucleótido del inicio del gen. Este sitio facilitó la transferencia de los genes hPL a pNUT. Para proveer a hPL-1 con este sitio BamHI conveniente para las manipulaciones, Ramiro Ramírez-Solís en trabajos previos en nuestro laboratorio realizó la construcción de un gen híbrido entre hPL-1 y hPL-3 (111). Este nuevo gen consistió del primer exón, primer intrón y parte del segundo exón del gen hPL-3 y posee intactas las secuencias hPL-1 desde el sitio PvuII, en el segundo exón, hasta el sitio EcoRI, al final del gen. Las nuevas secuencias al inicio del gen hPL-1, solo difieren de las secuencias naturales en las posiciones aminoacídicas +10 y +11. Estas diferencias representan cambios de un residuo de ácido aspártico y otro de histidina en hPL-1, por residuos de lisina y ácido glutámico en hPL-3, respectivamente. Por lo que utilizamos este nuevo gen en lugar del gen hPL-1 natural.

Una vez que obtuvimos todos los plásmidos recombinantes con los genes hPL, caracterizamos sus DNAs mediante digestión con enzimas de restricción diagnósticas y realizamos una hibridación de DNA tipo Southern (94). En la figura 11 mostramos la anatomía molecular de los nuevos plásmidos, así como sus patrones de hibridación con la sonda radiactiva específica para los genes hGH y hPL (6). Todos los patrones de restricción resultaron como esperábamos y solo los fragmentos conteniendo los genes hibridaron con la sonda homóloga. Con lo anterior corroboramos la identidad de los diferentes genes de la familia hGH-hPL en el vector pNUT.

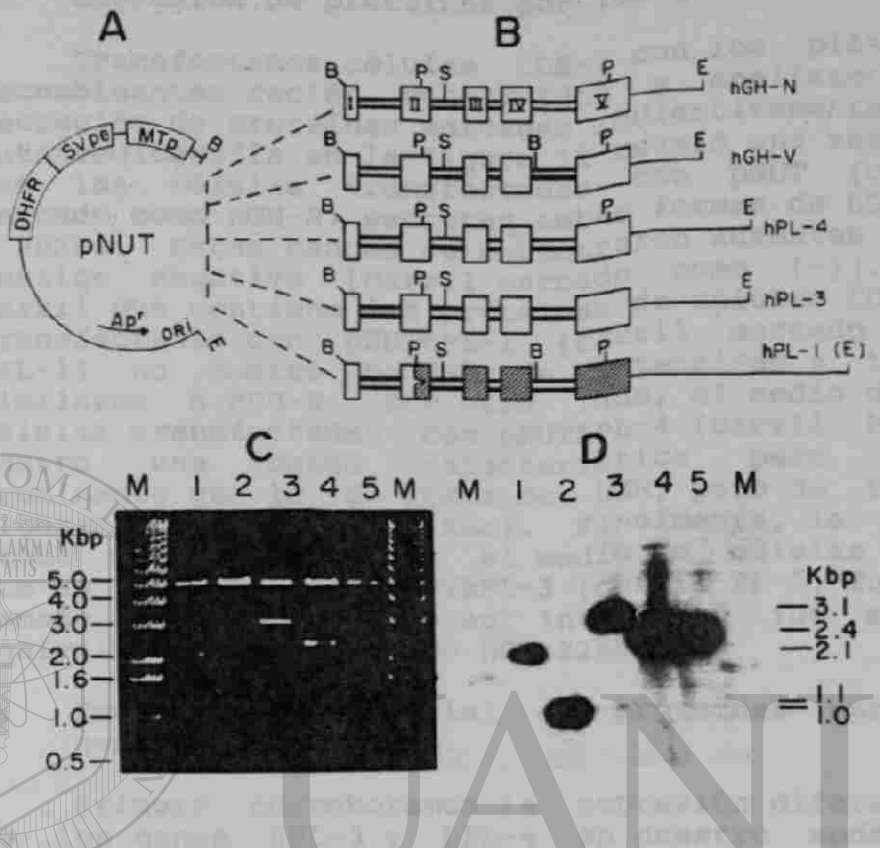

Figura 11. Construcción de los plásmidos de expresión para los genes hGH y hPL. En este diagrama esquematizamos la organización y mapa de los plásmidos de expresión (A y B). Para subclonar la región estructural de los genes en pNUT (A), reemplazamos la región hGH-N por la misma región de los otros genes. La digestión con las enzimas de restricción BamHI y EcoRI (C) así como la hibridación molecular con la sonda de hPL marcada radiactivamente (D) confirmaron la identidad de los nuevos plásmidos híbridos. En C y D los carriles contienen: 1, pNUT; 2, pNUThGH-V; 3, pNUThPL-1; 4, pNUThPL-3; y 5, pNUThPL-4. M corresponde a marcadores de peso molecular representados en kpb, (kbp). Los tamaños de las bandas que hibridaron con la sonda estan indicados en kpb (kbp) a la derecha de D, utilizamos el gen hPL-1 híbrido entre el gen hPL-3 y el hPL-1 (B) en lugar del gen hPL-1 natural. pNUThGH-V no fue empleado en este estudio. DHFR indica la unidad transcripcional para la dihidrofolato reductasa. SVpe representa al promotor-potenciador "enhancer" de SV40. MTp señala al promotor de la metalotioneína. B=BamHI; P=PvuII; S=SacI y E=EcoRI. Los exones se indican con números romanos y los intrones con línea doble.

B. Expresión de proteínas por los genes hPL.

Transfectamos células COS-7 con los plásmidos recombinantes recién construídos y analizamos la secreción de proteínas marcadas radiactivamente. La autorradiografía en la figura 12 reveló una vez mas que las células transfectadas con pNUT (carril marcado como hGH-N) secretan ambas formas de hGH, 22 y 20KDa. Estas bandas se encontraron ausentes en el testigo negativo [carril marcado como (-)]. El carril que contiene las proteínas de células COS-7 transfectadas con pNUThPL-1 (carril marcado como hPL-1) no mostró bandas de intensidad y tamaño similares a hGH-N. Por otro lado, el medio de las células transfectadas con pNUThPL-4 (carril hPL-4) mostró una banda característica pero menos prominente que la de 22KDa de hGH, pero de tamaño ligeramente mayor (~ 25KDa). Finalmente, la única banda que observamos en el medio de células a las que se les introdujo pNUThPL-3 (carril PL-3) fue del tamaño de hPL-4, pero su intensidad fue mayor, equivalente a la banda de hGH 22KDa.

C. Producción diferencial de proteínas por los genes hPL-3 y hPL-4.

Primero corroboramos la expresión diferencial de los genes hPL-3 y hPL-4 en nuestro modelo de cultivo celular (figura 13B). Posteriormente, construímos recombinantes híbridos entre hGH y hPL para analizar la expresión de los genes hPL-3 y hPL-4 (figura 13A). Detectamos diferencias en la abundancia relativa entre las proteínas quiméricas entre hPL-3/hGH versus hPL-4/hGH, expresadas por los genes híbridos correspondientes. De la misma forma que con los genes hPL no híbridos, observamos menos proteína cuando las secuencias de los primeros dos exones provienen de hPL-4 que cuando estas provienen de hPL-3. Estos resultados se muestran en la figura 13B en los carriles GH (PL-3:I,II) y GH (PL-4:I,II).

Por otro lado, en la construcción recíproca, donde los últimos tres exones fueron del gen hPL-4 y los primeros dos de hGH-N (figura 13A), la intensidad de la banda correspondió a aquella de hGH [figura 13B: carril PL-4(GH:I,II)]. Por lo tanto, la abundancia relativa de las proteínas naturales o quiméricas hGH-hPL dependió de los dos primeros exones, en nuestro modelo in vitro de cultivo celular.

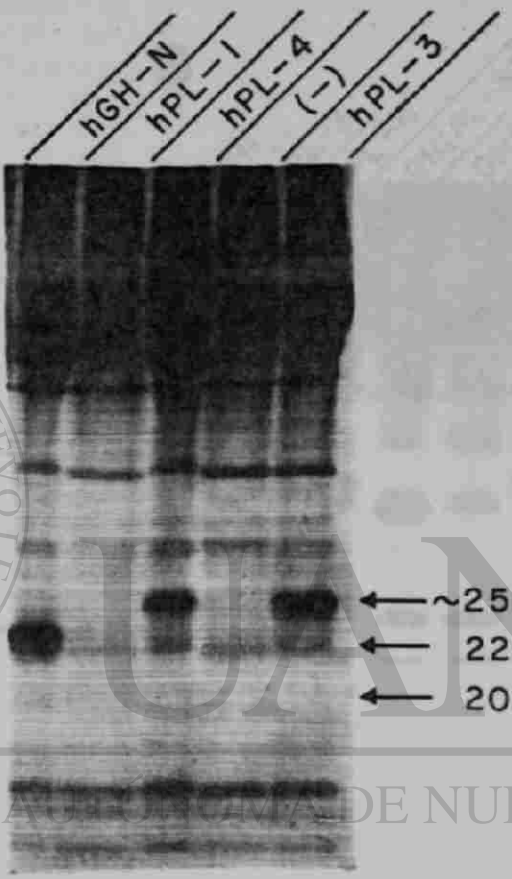

Figura 12. Producción <u>in vitro</u> de proteínas secretadas derivadas de los genes hPL. Analizamos mediante electroforesis en gel de poliacrilamida-SDS y autorradiografía el medio de las células COS-7 transfectadas con los plásmidos indicados en cada carril e incubamos en presencia de [^{35}S]-metionina. El símbolo (-) identifica al medio de células transfectadas con el vector solo pNUT(-). Los tamaños de las bandas hPL y hGH características se indican en KDa (K).

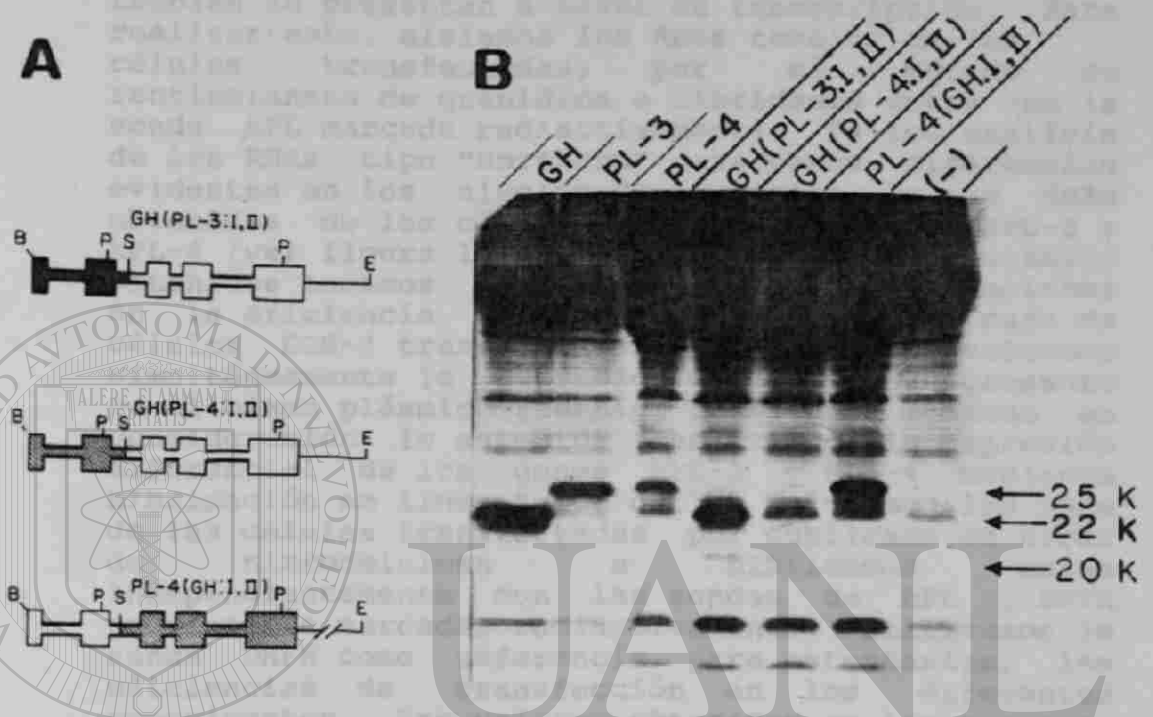

Figura 13. Diversidad en tamaño y abundancia de las proteínas codificadas por los genes hGH y hPL. Los genes recombinantes híbridos entre hGH y hPL que utilizamos en estos experimentos son mostrados en (A) para un mejor seguimiento y claridad de los resultados. El autorradiograma como en las figuras anteriores muestra el análisis de proteínas radiactivas secretadas por las células COS-7 transfectadas. Cada carril corresponde al medio de células transfectadas con los plásmidos conteniendo los genes indicados arriba (B). Las bandas nuevas se señalan con flechas y su tamaño también se muestra en KDa (K).

D. Origen transcripcional de la expresión diferencial de los genes hPL-3 y hPL-4.

Decidimos analizar si las diferencias en expresión de los genes hPL-3 y hPL-4 detectadas también se presentan a nivel de transcripción. Para realizar esto, aislamos los RNAs totales de las células transfectadas, por el método de isotiocianato de guanidina e hibridamos éstos con la sonda hPL marcada radiactivamente. En los análisis de los RNAs tipo "Northern" detectamos diferencias evidentes en los niveles de expresión de los RNAs obtenidos de las células transfectadas con hPL-3 y hPL-4 (ver figura 14). Para validar los resultados obtenidos tomamos en consideración las variaciones en la eficiencia de transfección en cada caja de células COS-7 transfectadas. Para ello evaluamos simultáneamente la expresión del gen DHFR presente en el mismo plásmido (testigo interno). Tomando en consideración lo anterior analizamos la expresión diferencial de los genes hPL-3 y hPL-4 mediante hibridación en línea "slot dot". Colocamos los RNAs de las células transfectadas por duplicado en tiras de nitrocelulosa e hibridamos estas independientemente con las sondas de hPL y DHFR previamente marcadas radiactivamente. Utilizamos la sonda DHFR como referencia para estandarizar las eficiencias de transfección en los diferentes experimentos. Los valores obtenidos en las tiras de RNAs hibridadas con la sonda DHFR fueron utilizados para normalizar las cantidades de RNAs que se depositaron en otro experimento donde corregimos las diferencias en eficiencias de transfección detectadas. En el autorradiograma de la figura 15 se muestra que logramos normalizar y tener una señal constante cuando hibridamos con la sonda radiactiva DHFR y señales notablemente diferentes para el caso de la sonda de hPL. Cuantificamos las diferencias de expresión mediante espectofometría de centelleo líquido y detectamos una disminución de ocho veces en la expresión de los RNAs de hPL-4 en relación a los de hPL-3. Con lo anterior demostramos que la expresión diferencial entre las proteínas hPL-3 y hPL-4, depende cuando menos en parte de diferencias en los niveles de los RNAs correspondientes.

E Efecto de la mutación al inicio del segundo intrón de hPL-1 en la producción de proteínas.

Analizamos también el efecto de la mutación del intrón de hPL-1 en la producción de proteínas. Nos

Figura 14. Expresión de los genes hPL-3 y hPL-4 a nivel de RNAm. Obtuvimos los RNAs totales de las células COS-7 transfectadas con los plásmidos recombinantes por el método de isotiocianato-fenol-cloroformo. La autorradiografía muestra los resultados del análisis tipo "Northern" de los RNAs obtenidos de las células transfectadas con los plásmidos pNUThPL-3 (carril PL-3) y pNUThPL-4 (carril PL-4). P identifica al carril que contiene RNAs totales de hipófisis humanas.

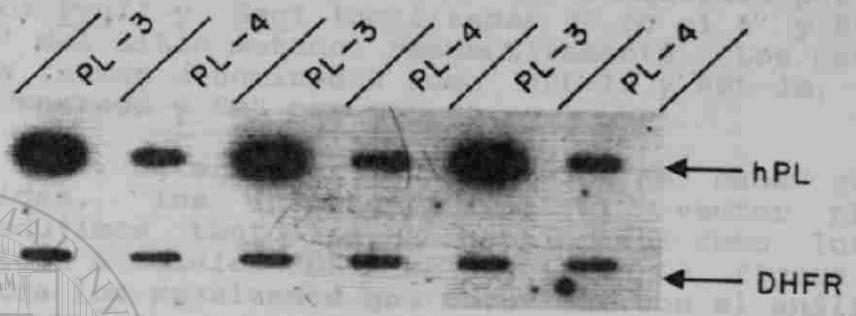

Figura 15. Análisis de la expresión diferencial de los genes hPL-3 y hPL-4 mediante hibridación en línea "slot dot". 3 µg de los RNAs que obtuvimos de las células transfectadas con los plásmidos recombinantes pNUT conteniendo los genes hPL-3 y hPL-4 fueron hibridados independientemente con sondas de hPL y DHFR previamente marcadas radiactivamente. En la autorradiografía mostramos que logramos normalizar y tener una señal constante cuando hibridamos con la sonda radiactiva DHFR y señales notablemente diferentes para el caso de sonda de hPL. Cuantificamos las diferencias de expresión mediante espectrofotometría de centelleo líquido.

basamos en trabajos previos realizados en nuestro laboratorio por Ramiro Ramírez-Solís (106), que iniciaron los estudios a nivel de RNA para probar si la mutación al inicio del segundo intrón del gen hPL-1 era la única causa de su aparente inactividad. En estos experimentos se intercambiaron las secuencias nucleotídicas que incluyen el borde entre el segundo exón y segundo intrón de los genes hPL-1 y hPL-3. Estas secuencias están flanqueadas por los sitios PvuII y SacI localizados 30 pb al 5' y 86 pb al 3' del sitio mutado, respectivamente. Los nuevos genes fueron denominados como hPL-1r y hPL-3m, "r" por reparado y "m" por mutado.

Para determinar la expresión de esos genes híbridos, los insertamos en el vector pNUT. Introdujimos tanto los genes híbridos como los no híbridos a células COS-7 en cultivo. La figura 16B muestra los resultados que obtuvimos con el análisis de proteínas marcadas en las células transfectadas. Como se puede apreciar en los carriles PL-3 y GH, detectamos fácilmente bandas de proteínas con los tamaños esperados en los medios de células transfectadas ya sea con pNUThPL-3 o pNUT. Sin embargo, el medio de células a las que les introdujimos pNUThPL-1 (carril PL-1) o pNUThPL-3m (carril PL-3m) no mostraron bandas distintas a las del testigo negativo. Por lo tanto, aún después de reparar el gen hPL-1, no pudimos observar la expresión de una nueva banda proteica (carril PL-1r). Interesantemente, la introducción de la mutación del segundo intrón de hPL-1, al gen hPL-3 inactivó la expresión de este último (carril PL-3m).

Corroboramos los resultados anteriores cuando construímos recombinantes híbridos entre hGH y hPL (figura 16A) e introdujimos estos a células COS-7. La expresión y secreción del recombinante GH(PL-3:I,II) mostró una banda prominente parecida en tamaño e intensidad a la obtenida con el gen hGH (ver figura 16B). Pero con el recombinante que contiene la mutación GH(PL-3m:I,II) no detectamos banda prominente [figura 16B:carril GH(PL-3:I,II)]. En el caso del recombinante GH(PL-3:I,II) también se produjo, al igual que con hGH, la segunda banda (20KDa) de menor tamaño e intensidad. Con esto demostramos que en su forma de gen híbrido, también funcionan las secuencias del gen hGH-N responsables de la vía alternativa de procesamiento del RNAm que codifica para la forma de hGH 20KDa.

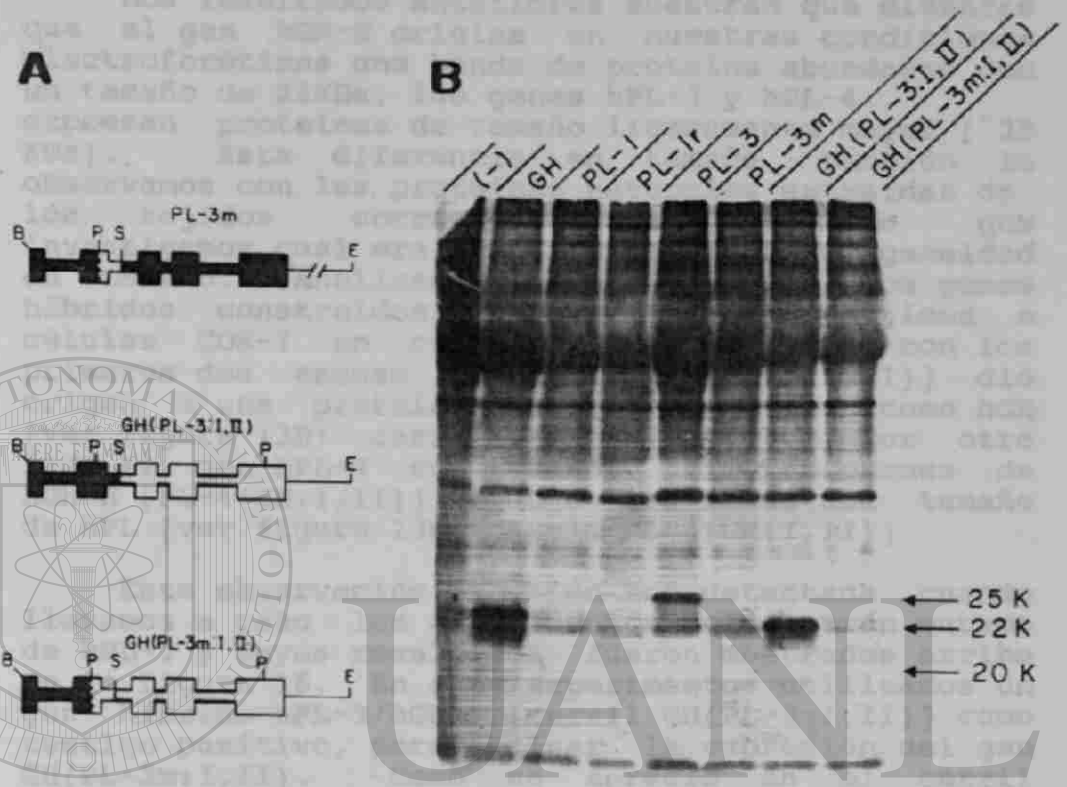

Figura 16. Efecto de la mutación al inicio del segundo intrón del gen hPL-1 sobre la producción de proteínas secretadas. Los genes híbridos descritos en (A) fueron construídos al unir, a través del sitio único SacI en el segundo intrón, los primeros dos exones de los genes hPL-3 (natural) y hPL-3m (mutado) con los últimos tres exones del gen hGH-N. Medios de cultivo de células transfectadas con los diferentes genes híbridos (indicados arriba) fueron analizados para determinar la presencia de proteínas radiactivas secretadas (B). Las flechas indican el tamaño en KDa (K) de las nuevas proteínas originadas por los genes introducidos transitoriamente.

F Diferencias aparentes en los tamaños de hPL y hGH producidas por los genes.

Los resultados anteriores muestran que mientras que el gen hGH-N origina en nuestras condiciones electroforéticas una banda de proteína abundante con un tamaño de 22KDa, los genes hPL-3 y hPL-4 expresan proteínas de tamaño ligeramente mayor (~25 KDa). Esta diferencia en tamaño también la observamos con las proteínas naturales extraídas de los tejidos correspondientes, por lo que investigamos cual era la causa de tal heterogeneidad en tamaño. Analizamos la expresión de los genes híbridos construídos, cuando los introdujimos a células COS-7 en cultivo. El gen hGH con los primeros dos exones de hPL-4 [GH(PL-4:I,II)] dió origen a una proteína que se comportaba como hGH [ver figura 13B: carril GH(PL-4:I,II)]. Por otro lado el gen hPL-4 con los dos primeros exones de hGH-N [PL-4(GH:I,II)] produjo una banda del tamaño de hPL [ver figura 13B; carril PL-4(GH:I,II)].

Este observación también fué detectada cuando llevamos a cabo los experimentos del intrón mutado de hPL-1 y cuyos resultados fueron mostrados arriba en la figura 16. En esos experimentos utilizamos un gen híbrido hPL-3/hGH-N [carril GH(PL-3:I,II)] como testigo positivo, para evaluar la expresión del gen GH(PL-3m:I,II). Como se aprecia en el carril marcado como GH(PL-3:I,II), la proteína secretada derivada de este gen híbrido presentó como hGH, el tamaño de 22KDa por lo que se refiere al tamaño (22 KDa).

Los resultados que obtuvimos en los experimentos descritos en esta sub-sección fueron utilizados para publicar el artículo: Coding potential of transfected human placental lactogen genes y fueron incluídos en la revisión: Transcriptional regulation of human placental lactogen genes (anexo B).

III Disección <u>in vitro</u> de los genes hPL mediante mutagénesis por DNA homológo.

Para cuantificar la expresión diferencial de los genes hPL descrita en la sección anterior diseñamos una estrategia que denominamos "Mutagénesis por DNA homólogo". Esta estrategia consiste en insertar las regiones de interés de algún gen homólogo en el contexto del gen que codifica para hGH. Para realizar

esto nos basamos en la alta similitud existente en las secuencias aminoacídica y nucleotídica de hGH y hPL.

A Diferencias cuantitativas en la expresión de los genes hPL-3 y hPL-4.

Utilizamos esta estrategia de mutagénesis por DNA homólogo para reemplazar un segmento que comprende las primeras 588 pb del gen, desde el sitio del casquete al inicio de la transcripción hasta el segundo intrón del gen hGH, por los segmentos correspondientes de los genes hPL-3 y hPL-4 (figura 17). Insertamos estos genes híbridos en el vector pNUT (-) y caracterizamos los plásmidos recombinantes híbridos resultantes mediante digestión con enzimas de restricción diagnósticas. Estos segmentos de 588 pb de los genes hPL activos presentan solamente ocho cambios nucleotídicos distribuídos como sigue: uno en la región del extremo 5' no traducible, otro en el primer exón (péptido señalador), tres en el primer intrón y tres en el segundo intrón. El hecho de que las proteínas quiméricas GH(PL-3:I,II) y GH(PL-4:I,II) contienen la mayor parte de los aminoácidos (169 de 191) de hGH, nos permitió cuantificar estas proteínas quiméricas mediante RIA para hGH.

Primeramente realizamos tres preparaciones independientes de DNA de los plásmidos recombinantes híbridos mediante la técnica de preparación a gran escala. De tal forma que obtuvimos los plásmidos en las mismas condiciones para evitar variaciones en las eficiencias de transfección debidas a la calidad de los DNAs plasmídicos.

Estos tres grupos de plásmidos recombinantes fueron utilizados independientemente para tranfectar células COS-7 mediante la técnica de precipitación con fosfato de calcio-DNA. En estos experimentos realizamos co-transfección con el plásmido pCMcat para medir la actividad de la enzima CAT y determinar así la eficiencia en los experimentos de transfección. 48 h después colectamos el sobrenadante de las células transfectadas y cuantificamos la hGH presente en el medio extracelular. Además determinamos la actividad de CAT en los extractos de las células co-transfectadas (figura 18) y utilizamos estos resultados para normalizar las proteínas quiméricas sintetizadas por las células transfectadas con los plásmidos recombinantes. En estos experimentos encontramos

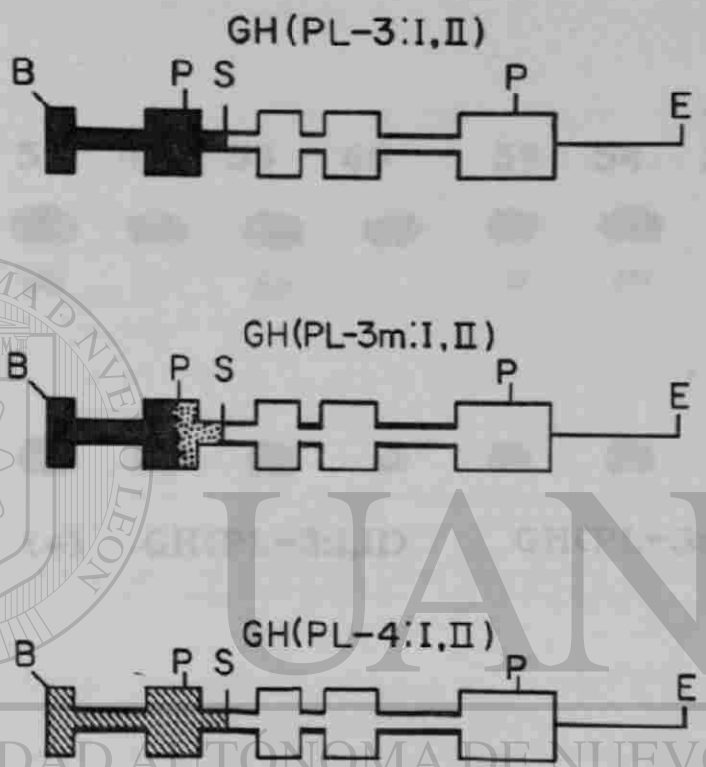

Figura 17. Organización de las construcciones génicas quiméricas. El diagrama ilustra los mapas y origen de los fragmentos génicos usados para las construcciones quiméricas hGH-hPL (2150pb). Los sitios de corte para las enzimas de restricción estan indicados como B=BamHI, P=PvuII, S=SacI y E=EcoRI. El origen de los fragmentos se muestra en negro para hPL-3, punteado para hPL-1, rayado para hPL-4 y blanco para hGH.

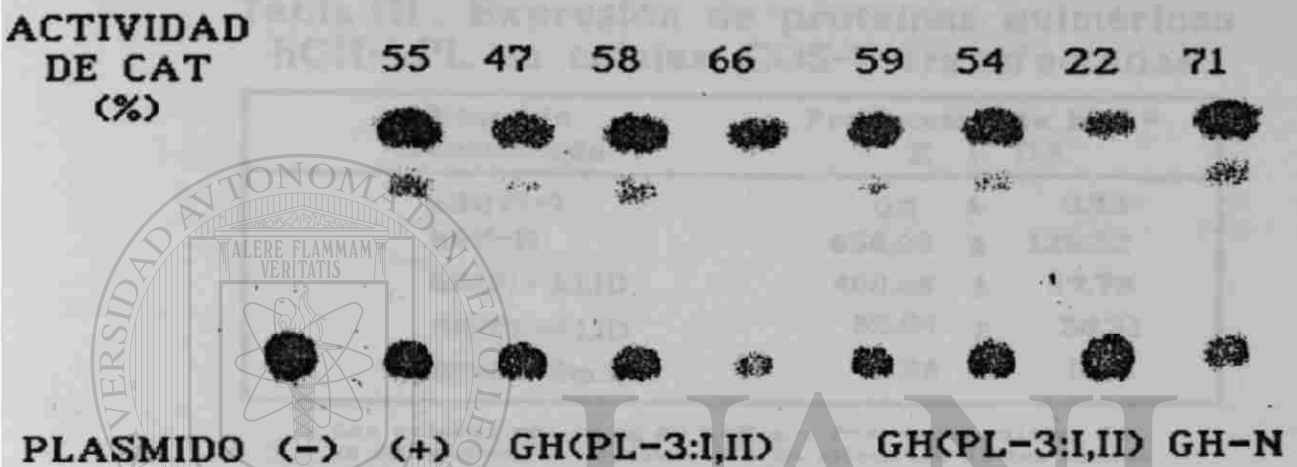

Figura 18. Comparación de la eficiencia de expresión de los plásmidos recombinantes híbridos hGH-hPL en células COS-7. Introdujimos los plásmidos recombinantes GH(PL-3:I,II) y GH(PL-4:I,II) en las células COS-7 mediante la técnica de co-precipitación con fosfato de calcio-DNA. 48h después determinamos en los extractos celulares la cantidad de proteínas por el método de Bradford y analizamos la actividad de CAT. El autorradiograma muestra ligeras diferencias en la actividad CAT con los diferentes plásmidos recombinantes. Como estos resultados son cualitativos, cortamos en la placa de sílica-gel la región que contenía las formas acetiladas de cloranfenicol marcado con [^{14}C] (bandas superiores) y cuantificamos la radiactividad presente mediante espectofometría de centelleo líquido (Actividad de CAT).

que el plásmido recombinante híbrido GH(PL-3:I,II) secreta cinco veces más proteína que GH(PL-4:I,II) (datos incluidos en la tabla III). Encontramos que el medio de las células transfectadas con el plásmido que contiene los dos primeros exones de hPL-3 [GH(PL-3:I,II)] contenía una cantidad de hGH equivalente al 62.3% de la detectada en el testigo positivo (ver tabla III).

Tabla III. Expresión de proteínas quiméricas hGH-hPL en células COS-7 transfectadas.

Plásmido transfectado	Producción de hGH * X ± D.S.
pNUT(-)	0.3 ± 0.73
hGH-N	656.03 ± 126.32
GH(PL-3:I,II)	408.68 ± 69.79
GH(PL-4:I,II)	82.94 ± 30.31
GH(PL-3m:I,II)	0.28 ± 0.68

* Los valores son en ng de hGH por frasco de cultivo de 75 cm^2 obtenidos mediante RIA en alicuotas de los medios y corresponden al promedio ($\bar{X}$) y desviación estandard (D.S.) de tres experimentos independientes.

De estos resultados podemos deducir una vez más que en los dos primeros exones de hPL-3 y hPL-4 se encuentran codificadas las características que conducen a las diferencias en expresión de los genes hPL-3 y hPL-4.

B Cuantificación del efecto de la mutación del gen hPL-1.

En otros experimentos construímos genes híbridos hGH cuyos dos primeros exones y parte del segundo intrón corresponden ya sea a hPL-3 o hPL-3m. Estos nuevos genes híbridos GH(PL-3:I,II y GH(PL-3m:I,II), consisten de secuencias de hPL-3 o hPL-3m desde el inicio del gen hasta el segundo intrón (en el sitio único SacI) a las que les unimos secuencias del gen hGH-N, desde su sitio SacI en el segundo intrón, hasta el sitio EcoRI al final del gen (ver figura 17).

Cuando introdujimos éstos genes híbridos y sus plásmidos testigos a células COS-7 en cultivo

detectamos las proteínas quiméricas derivadas de la expresión de estos genes y mediante RIA para hGH. Los niveles de hGH en el medio de las células transfectadas con el plásmido que contiene el gen híbrido hPL-3m/hGH-N [plásmido:GH(PL-3mI,II)] no fueron detectados por RIA (ver tabla III). Estos resultados con las proteínas quiméricas corroboran que la mutación en el sitio donador del procesamiento de intrón al inicio del segundo intrón del gen hPL-1, podría ser una de las causas de la falta de expresión de este gen, ya que inactivó totalmente la expresión de la proteína quimérica GH(PL-3,I,II).

Utilizamos los resultados que obtuvimos en los experimentos descritos en esta sub-sección para publicar el artículo: Expression studies of transfected multigene families by homologous DNA mutagenesis (anexo C).

DISCUSION Y CONCLUSIONES

La reintroducción a células en cultivo, de genes aislados por técnicas de Ingeniería Genética (94, 96), ha resultado ser un método de gran valor en la identificación y disección de secuencias nucleotídicas requeridas para el control del funcionamiento del gen. También ha brindado valiosa información sobre los efectos de mutaciones en estas secuencias. Escogimos esta metodología para determinar el potencial de codificación de todos los genes hPL.

Los estudios de clonación de DNA y de determinación de secuencias nucleotídicas han conducido al aislamiento de clonas de DNAc para todos los genes de la familia hGH-hPL, excepto hPL-1. Una gran cantidad de información referente a la expresión de genes hGH y hPL resultó de estos estudios de DNAc. A pesar de estos, pocos han sido los experimentos encaminados a demostrar que algún RNAm particular de los genes hGH efectivamente se traduce en proteínas. Para el caso de los genes hPL esta clase de experimentos simplemente son inexistentes.

Cuando introdujimos los genes hPL activos a la línea celular COS-7 de riñón de mono, bajo el control de sus promotores naturales, no pudimos detectar señal en un análisis de RNA tipo "Northern" de los transcritos. Esta falta de expresión puede deberse a la ausencia en estas células de factores de transcripción específicos para los genes hPL. También puede explicarse por un requerimiento del amplificador de la transcripción del gen hPL (40), o por ambas razones. Se ha demostrado que se requiere la presencia de un factor transcripcional particular, llamado GHF-1, para alcanzar una expresión eficiente del gen hGH-N (110). Los resultados que obtuvimos usando los promotores naturales de los genes estan en concordancia con esta observación. Como también lo está el hecho de haber conseguido expresar las secuencias estructurales de estos genes al fusionarlas a señales transcripcionales potentes y de especificidad celular amplia.

Para la ejecución del presente trabajo, primeramente desarrollamos un sistema eficiente para detectar la expresión de genes en cultivo celular. Para realizar esto, diseñamos y construímos vectores eucarióticos de expresión y comparamos estos vectores con otros comunmente usados para seleccionar los más potentes para dirigir la expresión de hGH en cultivo celular. Además, llevamos a cabo un análisis funcional

de las hGHs de 20 y 22KDa producidas in vitro en el modelo de diferenciación de fibroblastos 3T3 hacia adipocitos. Posteriormente analizamos el potencial de codificación in vitro de los genes hPL. Además, diseñamos una estrategia que denominamos "Mutagénesis por DNA homólogo" que nos permitió cuantificar la expresión diferencial de los genes hPL-3 y hPL-4 y analizar el efecto de la mutación presente al inicio del segundo intrón del gen hPL-1.

El modelo experimental que desarrollamos en este trabajo consiste en la detección de la expresión de genes y DNAcs en cultivo celular. Primeramente insertamos la secuencia estructural del gen hGH bajo la influencia de la unidad promotor-potenciador del hCMV. Cuando introdujimos este plásmido a células COS-7 en cultivo, demostramos que la hGH producida in vitro por las células COS-7 es secretada al medio extracelular, lo cual esperábamos ya que hGH es una proteína normalmente secretada por las células de la hipófisis. Estos resultados nos permitieron concluír que el vector pAVE1hGH es capaz de expresar y secretar hGH eficientemente en cultivo de células eucarióticas.

Posteriormente comparamos la expresión de este vector con otros vectores de expresión comunmente utilizados. Para ello, analizamos la expresión de hGH en las células COS-7 transfectadas tanto a nivel de RNAm (datos no mostrados) como a nivel de proteínas secretadas al medio extracelular. En ambos casos detectamos los mayores niveles de expresión en las células transfectadas con los plásmidos pAVE1hGH y pNUT. Estos valores de hGH son muy similares a los detectados por Selden y col. en células L transfectadas transitoriamente (52) y son cuarenta veces mayores que los obtenidos en células Vero transfectadas con vectores basados en SV40 (47). Por otro lado, determinamos la capacidad de estas hGHs producidas in vitro para inducir específicamente la conversión de fibroblastos 3T3 hacia adipocitos. La conversión adipocítica dependió de la cantidad de hGH adicionada, por lo que obtuvimos la mayor actividad funcional de las hGHs secretadas por las células transfectadas con los plásmidos pNUT y pAVE1hGH (resultados no mostrados), en comparación con los otros vectores arriba mencionados.

Después que detectamos reproduciblemente la expresión del gen estructural hGH en cultivo celular, analizamos la expresión de los DNAcs de hGH obtenidos en trabajos previos en nuestro laboratorio. Detectamos

niveles bajos de expresión del DNAc de hGH 20KDa en relación al DNAc de hGH 22KDa. En estudios previos realizados en nuestro laboratorio mediante análisis de hibridación tipo "Northern" de los RNAs de las células transfectadas con estos mismos plásmidos mostraron que las señales de hibridación dadas por los RNAs expresados, tanto por pAVEhGH22K como pAVEhGH20K, fueron similares en intensidad. Estos resultados indicaron que ambos DNAcs son eficientes en la producción de RNAms maduros (109). Además se ha demostrado que el gen hGH expresado transitoriamente, produce niveles de RNAm equivalentes a los encontrados por nosotros y que los niveles de expresión de RNAm reflejan directamente los niveles de proteína (52). Por lo tanto, dado que no teníamos evidencias para dudar de la integridad e identidad de nuestros genes, establecimos como posible explicación para tan sorprendentemente bajos niveles de RIA de hGH 20KDa, el que los anticuerpos del estuche comercial para RIA de hGH que usamos, al ser altamente específicos para hGH22KDa, reconocen pobremente la variante de 20KDa de la hormona. Nuestra suposición fué apoyada por los hallazgos de que la variante 20KDa presenta únicamente un 20 a un 50% de capacidad para desplazar [^{125}I]-hGH unida a anticuerpo, así como un 30 a 50% de capacidad para desplazar [^{125}I]-hGH unida al receptor de membrana de hígado de rata o conejo (111).

En vista de lo anterior fué necesario demostrar que era posible visualizar la expresión de las diferentes formas conocidas de hGH. Cuando analizamos la cantidad de proteínas presentes en el medio extracelular de las células COS-7 transfectadas, encontramos que aunque los niveles de expresión transitoria de hGH en nuestros cultivos resultaron altos (hasta 300 ng/ml), eran superados por los niveles de proteínas totales presentes como componentes del medio de cultivo (2mg/ml). Esto impedía la visualización de la banda proteica correspondiente a hGH al teñir las proteínas separadas en el gel. Una complicación adicional resultó ser la presencia de bGH, porque presenta un tamaño muy similar a hGH y hPL y es un componente del SFT que utilizamos para complementar el medio de cultivo. Tampoco consideramos conveniente utilizar técnicas de detección como la inmunoelectrotransferencia para detectar las proteínas de interés, debido a que contábamos solamente con los anticuerpos contra las formas mejor conocidas de hGH y hPL (22KDa). Por lo que con esta limitante tendríamos que confiar en una buena reacción cruzada para detectar variantes proteicas que pudieran derivarse de todos los

genes hGH y hPL. Decidimos entonces realizar un marcaje de las proteínas sintetizadas de novo por las células transfectadas con [^{35}S]-metionina. En estos experimentos detectamos bandas correspondientes a las hGHs de 22KDa y 20KDa con las dos concentraciones de metionina utilizadas. Obviamente fué necesario asegurarnos que las bandas de proteínas correspondieran con los tamaños esperados y que su presencia coincidiera con la transfección del plásmido que contiene el gen que la codifica. La abundancia de la hGH de 20KDa, estimada por apreciación visual, coincidió con la abundancia de la variante en la glándula pituitaria, donde el 90% de la producción de hGH es de 22KDa y el 10% corresponde a la forma de 20KDa (57, 111).

Ya que seleccionamos la concentración menor de metionina marcada para realizar el marcaje, logramos visualizar las proteínas secretadas por los DNAcs de hGH. En estos experimentos detectamos la expresión de las hGHs de 20 y 22KDa. Aunque la expresión de ambas formas de hGH presenta diferencias en la intensidad de las bandas pudimos demostrar la ineficiente detección inmune del anticuerpo específico de la hGH 22KDa por la variante de 20KDa. Por otro lado, logramos verificar la actividad biológica de ambas formas de hGH en la conversión adipocítica de fibroblastos 3T3. Consideramos con lo anterior que nuestro modelo estaba adaptado para el análisis de la expresión de genes y DNAcs en cultivo celular.

Una vez que implementamos el modelo experimental, analizamos la expresión de todos los genes hPL en cultivo celular. Estos resultados nos permitieron determinar, por primera vez, la expresión in vitro y el potencial de codificación de todos los genes hPL. Los resultados que aportamos demuestran que los genes hPL-3 y hPL-4, mas no hPL-1, contribuyen a la producción de hPL madura. También demostramos que a pesar de ser altamente similares, las secuencias estructurales, de estos genes responden diferencialmente al mismo promotor heterólogo. Aunque las proteínas expresadas por estos genes son idénticas en su estructura primaria, difieren en el nivel de expresión. Como consecuencia de este hallazgo, diseñamos una estrategia que denominamos "mutagénesis por DNA homólogo". Esta consistió en la inserción de regiones mutadas de genes homólogos en el contexto del gen que codifica para un producto fácilmente cuantificable.

Demostramos la factibilidad de esta estrategia

mediante la sustitución de segmentos del gen hGH-N, por secuencias homólogas del gen hPL, que nos permitieron el análisis de mutaciones sin afectar la conformación nativa de la proteína modificada. Analizamos el efecto de dos tipos diferentes de mutaciones presentes en los genes hGH y hPL. Para la primera escogimos la variación de secuencias existentes entre los primeros dos exones de los genes hPL-3 y hPL-4. La segunda correspondió a la mutación puntual del pseudogen hPL-1, antes referida y presente al inicio de su segundo intrón.

Para analizar la primera mutación intercambiamos los dos primeros exones de los genes hPL-3 y hPL-4 con los del gen hGH-N. Encontramos una disminución del 80% en los niveles de expresión de hPL-4 comparados con los de hPL-3. Pudiera argumentarse que los resultados anteriores solo indican una disminución en el reconocimiento de ambas proteínas quiméricas hPL/hGH por los anticuerpos específicos del RIA para hGH. Sin embargo consideramos que nuestro enfoque es adecuado y los resultados son válidos para detectar efectos de las diferencias nucleotídicas sobre la expresión génica ya que las secuencias aminoacídicas de estas proteínas quiméricas secretadas al medio de cultivo son idénticas. Los cambios se encuentran en las formas precursoras o inmaduras de estas proteínas quiméricas que presentan una diferencia aminoacídica en la tercera posición del péptido señalador. Esta diferencia podría influenciar la eficiencia de eliminación del péptido señal de estas proteínas. *Sin embargo*, cuando analizamos la expresión diferencial de los genes hPL-3 y hPL-4 a nivel de RNA detectamos una disminución de ocho veces la expresión, por lo que la acumulación diferencial extracelular de las proteínas hPL-3 y hPL-4 maduras se debe principalmente a diferencias en la eficiencia de transcripción de estos genes en nuestro modelo in vitro. Como ya hemos descrito los estudios in vivo en placenta humana de los niveles de RNA provenientes de hPL-3 y hPL-4 presentan mucha variabilidad y muestran una relación inversa a la que detectamos en nuestro modelo in vitro de cultivo celular donde la expresión esta dirigida por un mismo promotor heterólogo en relación a sus diferentes promotores y potenciadores naturales (6, 8, 23).

Además, encontramos que al introducir el área mutada de hPL-1 al gen hPL-3, la producción de la proteína hPL-3 secretada fué nula. A pesar de que reemplazamos la mutación al inicio del segundo intrón en hPL-1 por secuencias no-mutadas, no pudimos observar

proteína hPL-1 alguna, en el medio de cultivo de las células transfectadas con este gen reparado (hPL-1r). Por lo tanto, pensamos que deben existir mutaciones adicionales que contribuyen a esta carencia de expresión génica. Obtuvimos los mismos resultados cuando construímos genes hibridos entre los primeros exones de hPL-3 y hPL-3m con los últimos exones del gen HGH y cuantificamos los resultados mediante radioinmunoensayo. Evidencia adicional del efecto negativo de este tipo de mutaciones, al inicio de intrones, proviene también de estudios practicados in vitro. En tales estudios se introdujeron mutaciones al inicio de uno de los intrones del gen de ß-globina por mutagénesis dirigida por oligonucleótidos (112). En todos los casos estudiados a la fecha ya sea in vivo o in vitro, un cambio de guanosina monofosfato por adenosina monofosfato (G por A) al inicio del intrón, produce RNA mensajeros precursores incapaces de eliminar correctamente sus intrones.

Estos resultados también nos indicaron que la diferencia en tamaños entre las proteínas hGH y hPL, está codificada por los últimos tres exones de los genes que codifican para los aminoácidos 23 al 191 de la proteína. No tenemos explicación para las diferencias en tamaño observadas en el análisis electroforético de proteínas hPL y hGH. La diferencia la observamos tanto con las proteínas naturales, extraídas de sus tejidos humanos de origen, como con las proteínas expresadas por genes hGH y hPL transfectados. Puede ser posible que las diferencias aparentes de los tamaños observados correspondan simplemente a nuestras condiciones electroforéticas que permiten diferencias en el grado de interacción de las proteína y el SDS, y por lo tanto de desnaturalización de las proteínas.

Los resultados que obtuvimos nos permitieron llegar a las conclusiones abajo incluídas:

- Los genes hPL-3 y hPL-4 contribuyen a la producción de la hormona hPL madura en una relación de 4 a 1 en nuestro modelo in vitro.

- La expresión diferencial in vitro de los genes hPL-3 y hPL-4 depende en parte de las diferencias nucleotídicas presentes en los dos primeros exones de estos genes.

- La mutación al inicio del segundo intrón del gen hPL-1 es una de las causas que afectan su falta de

expresión, pero no la única, ya que no detectamos su reactivación cuando ésta fue reparada.

Además consideramos que uno de los logros mas importantes de este trabajo son las siguientes contribuciones:

- Desarrollamos un modelo experimental eficiente y confiable para detectar la expresión de genes y DNAcs en cultivo celular.

- Demostramos y cuantificamos la actividad adipogénica de los productos de expresión del gen hGH-N y sus DNAcs, en cultivo celular.

- Diseñamos un nuevo enfoque que denominamos "Mutagénesis por DNA homólogo" para la cuantificación de la expresión genética en familias multigénicas donde el producto de expresión de al menos uno de los miembros es cuantificable

Este nuevo enfoque permite determinar fácilmente el efecto en la expresión génica de cambios en secuencias nucleotídicas o mutaciones presentes en los miembros de la familia o de sus alelos. Con la utilización del gen que codifica para hGH, nuestro enfoque permite la cuantificación del efecto de prácticamente cualquier mutación o cambio en secuencia nucleotídica que ocurra en los otros genes hPL o hGH, miembros del complejo. Nuestro procedimiento podría ser usado con cualquier miembro de una familia multigénica que codifique para un producto que sea fácilmente cuantificable.

Los resultados obtenidos en este trabajo abren la posibilidad para realizar estudios encaminados a determinar si una, varias o la totalidad de las diferencias nucleotídicas presentes en los primeros exones de los genes hPL-3 y hPL-4 conducen a su diferente expresión y cuáles son las otras mutaciones en el gen hPL-1 involucradas en su falta de expresión. Por otro lado, el análisis de proteínas quiméricas hGH-hPL permitirá llevar a cabo un análisis de las relaciones estructura-función en el modelo de diferenciación de fibroblastos 3T3 hacia adipocitos e identificar el o los residuos aminoacídicos involucrados especificamente en esta actividad.

Las relaciones estructura-actividad se pueden estudiar basándose en la postulación de que los exones dentro de ciertos genes pueden codificar para dominios

funcionales importantes en la proteína codificada. Por lo tanto, la alteración por deleción o intercambio de exones íntegros mediante mutagénesis por DNA homólogo puede facilitar el mapeo de la o las regiones funcionales de la proteína en estudio.

Finalmente la producción estable de proteínas quiméricas hGH-hPL puede permitir el análisis estructural y/o funcional de otras actividades biológicas tales como la ganancia de peso en ratas hipofisectomizadas.

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ANEXO A

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New vectors for the efficient expression of mammalian genes in cultured cells

(Transient expression; cytomegalovirus enhancer-promoter unit; multiple cloning site; *hGH* gene and cDNA; polyadenylation site)

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SUMMARY

We have constructed a new pair of plasmid vectors for the efficient expression of mammalian genes. The first of the new plasmids, pAVE1, was derived from pCMVcat [Foecking and Hofstetter, Gene 45 (1986) 101–105] by replacing the chloramphenicol acetyltransferase-encoding sequences in the latter for a multiple cloning site. Since it possesses the powerful enhancer-promoter unit of the immediate early gene of human cytomegalovirus, pAVE1 is ideal for the expression of mammalian genes. The second expression vector, pAVE2, resulted when the 3′-end flanking region from the human growth hormone-encoding gene (*hGH*) was incorporated in pAVE1. This region provides sequences for 3′-end processing and polyadenylation of primary transcripts. Thus, pAVE2 is suitable for expression of cDNAs in cultured cells, where introns have little effect on gene expression. To test our new vectors, we inserted the structural region of the chromosomal *hGH* gene into pAVE1, and its cDNA into pAVE2. By independently transfecting the resulting recombinant plasmids into COS-7 cells, we have achieved high levels of *hGH* transient expression with both vectors.

INTRODUCTION

Bacterial plasmids modified by recombinant DNA techniques to facilitate expression of cloned genes or of their cDNAs in cultured cells are valuable tools for studies of eukaryotic gene structure and expression.

Earlier observations suggested that splicing was obligatory for mRNA accumulation in the cytoplasm (Hamer and Leder, 1979). Thus, the first generation of cDNA expression vectors usually included a heterologous intron in addition to the promoter and polyadenylation sequences (Mulligan and Berg, 1980). However, recent studies have shown that, in general, introns have little effect on the expression of genes transiently introduced into cultured cells (Brinster et al., 1988).

We searched for transcriptional control elements that could drive the expression in cultured cells of structural regions derived from cloned mammalian genes, more efficiently than the pair of expression vectors recently contributed by our laboratory (Cab-Barrera and Barrera-Saldaña, 1988). In a recent report, the hCMV enhancer-promoter unit was described as being one of the most powerful (Foecking and Hofstetter, 1986) and versatile units studied so far (Boshart et al., 1985). In the present work, we show the construction of a pair of new vectors

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Abbreviations: bp, base pair(s); CAT, Cm acetyltransferase; *cat*, gene encoding CAT; Cm, chloramphenicol; hCMV, human cytomegalovirus; hGH, human growth hormone; *hGH*, gene encoding hGH; hPL, human placental lactogen; kb, kilobase(s); MCS, multiple cloning site; nt, nucleotide(s); *ori*, origin of DNA replication; RIA, radioimmunoassay; SV40, simian virus 40.

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carrying this transcriptional control region. Their value for studies of gene expression is demonstrated here using them to transiently express the *hGH* gene sequences.

EXPERIMENTAL AND DISCUSSION

(a) Expression vectors

We compared the synthesis of CAT in COS-7 cells transfected separately with pSV2cat and pCMVcat (kindly provided by Dr. H. Hofstetter) using the DNA-Ca · phosphate co-precipitation technique (Graham and Van der Eb, 1973). The transfected cells were harvested for the CAT activity assay, 48 h after adding the precipitate (Gorman et al., 1982). As previously reported (Foecking and Hofstetter, 1986), we detected a much higher signal intensity from the acetylated forms of Cm produced by the extract of pCMVcat-transfected cells, as compared to that generated by the extract of those cells transfected with pSV2cat (data not shown). To exploit the advantages of the promoter strength and little tissue or species specificity (Boshart et al., 1985) provided by the hCMV enhancer-promoter unit, we decided to derive more versatile cloning and expression vectors from pCMVcat. The strategy for construction and the structure of the new expression vectors, pAVE1 and pAVE2, are illustrated in Fig. 1.

Vector pAVE1 was derived from pCMVcat by replacing the *Eco*RI-*Hin*dIII *cat* fragment with an analogous fragment of pUC19 MCS (Yanisch-Perron et al., 1985). This new plasmid harbors eight unique restriction sites (*Hin*dIII, *Sph*I, *Bam*HI, *Sma*I, *Xma*I, *Ava*I, *Kpn*I and *Eco*RI) immediately downstream from the hCMV enhancer-promoter unit. This MCS confers to pAVE1 great versatility for inserting and placing promoter-less chromosomal genes under the transcriptional control of the powerful hCMV enhancer-promoter unit.

Since introns have little effect on the expression of genes transfected into culture cells (Brinster et al., 1988), we decided to construct a second cloning vector for the transient expression of cDNA sequences. As illustrated also in Fig. 1, pAVE2 was derived from pAVE1 by incorporating the 3'-end flanking region from the *hGH* gene into the latter. This region includes sequences for primary transcript 3'-end processing and polyadenylation. Between the *hCMV* and *hGH* gene sequences, pAVE2 has six unique cloning sites: *Hin*dIII, *Sph*I, *Bam*HI, *Sma*I, *Xma*I and *Ava*I.

(b) Production of hGH by pAVE1 carrying the *hGH* gene

To test the ability of pAVE1 to express mammalian genes, we inserted the promoter-less chromosomal *hGH* gene, from a *Bam*HI site (at nt +2) to an *Eco*RI site (located about 600 nt downstream from the polyadenylation signal), between the same sites in pAVE1 (Fig. 1).

pAVE1hGH was transfected into COS-7 cells, and 48 later total RNA was isolated by the thiocyanate–phenol–chloroform technique (Chomczynski and Sacchi, 1987). Northern-blot analysis (McMaster and Carmichael, 1977; Thomas, 1980) of this RNA was carried out using a random primer-labeled (Feinberg and Vogelstein, 1983) *hPL* cDNA as a probe (Barrera-Saldaña et al., 1982). The probe displays over 90% sequence similarity to *hGH* cDNA. The autoradiograph showed an RNA band hybridizing to the *hPL* cDNA probe (data not shown) with the expected size for the *hGH* mRNA.

To verify the correct expression of the transfected novel hybrid gene, the production of secreted hGH by the transfected cells was determined using a commercially available (Diagnostic Products Co., Los Angeles, CA) hGH RIA kit. This test was performed directly on the tissue culture medium to determine the extracellular concentration of hGH. Values of secreted hGH averaged 1.2 μg/25-cm^2 flasks of transfected cells. Although using different cell lines, when compared with results reported in the literature, our values of hGH production are very similar to those obtained by transiently transfected L cells (Selden et al., 1986). Forty-fold lower values of hGH production have been reported for Vero cells transiently transfected with a SV40-based vector carrying the *hGH* gene (Lupker et al., 1983).

(c) Expression of *hGH* cDNA in culture cells using the pAVE2 vector

The pAVE2 vector is designed to be suitable for expressing cloned cDNAs because it carries nt sequences necessary for 3'-end processing and polyadenylation of primary transcripts. The ideal candidate to demonstrate the expression properties of pAVE2 was the *hGH* cDNA, since we had already expressed its corresponding gene using pAVE1 and thus comparisons could be made.

For the above purpose, and because we did not have available the *hGH* cDNA, we designed a simple and rapid method which allowed us to almost selectively clone the cDNAs for both the 22-kDa and 20-kDa forms of hGH, simultaneously. Details of this method will be described elsewhere (D.E. R.-L. and H.A. B.-S., in preparation). We then proceeded to introduce the *hGH* cDNA fragment corresponding to the 22-kDa form of hGH (hGH-22 K), into pAVE2. Since the cDNA copy we cloned is not full-length, we had to assemble an intron-less *hPL-hGH* hybrid gene. This gene was constructed using the following DNA fragments in a 5' → 3' direction: (*i*) the *hPL-3* gene (from the capping site to the *Pst*I site just before the end of the first exon); (*ii*) the *hPL-3* cDNA (from the *Pst*I site up to an *Aat*II site located at the beginning of the second exon); (*iii*) the *hGH* cDNA (from the *Aat*II site to the *Sma*I site

located 4 nt downstream from the stop codon); and (*iv*) the *hGH* gene from the *Sma*I site to the 3′ end of the gene (already present in the vector). In spite of this tailoring of gene pieces, the mature hGH protein derived from this hybrid gene is coded entirely by the *hGH* cDNA. The resulting hybrid was named pAVE2hGH22k.

After transfecting in parallel COS-7 cells with the pAVE2 derivative, as well as with the positive (pAVE1hGH) and negative (pAVE1) controls, we obtained total cellular RNAs and culture media and used them in Northern-blot

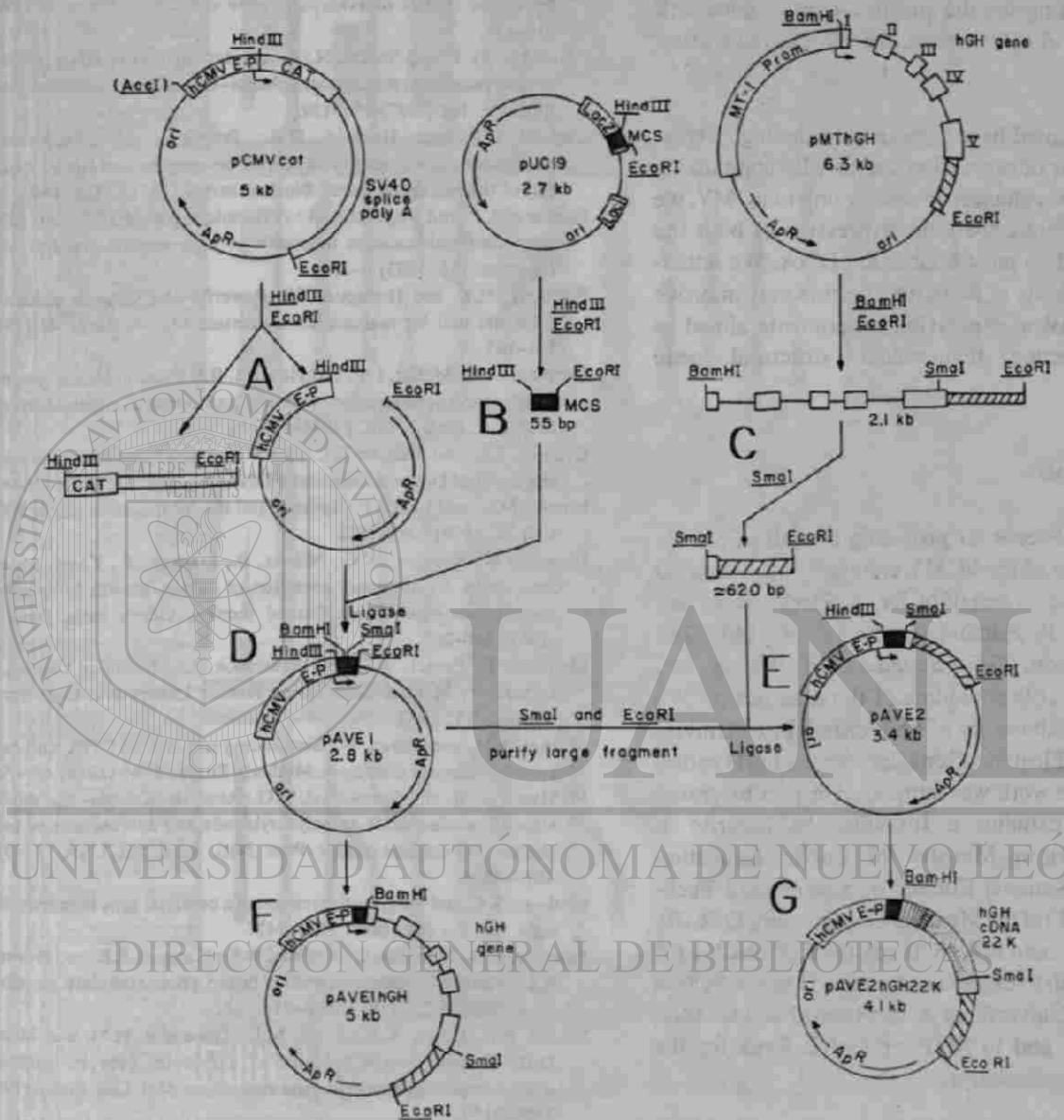

Fig. 1. Construction of the pAVE vectors and pAVEhGH derivatives. The plasmid pCMVcat (Foecking and Hofstetter, 1986), pUC19 (Yanisch-Perron et al., 1985) and pMThGH111 (Palmiter et al., 1983) were used for the construction of pAVE vectors and pAVEhGH derivatives. Restriction and modification enzymes were purchased from Bethesda Research Laboratories Inc. (Gaithersburg, MD), New England Biolabs Inc. (Beverly, MA) and International Biotechnology Inc. (New Haven, CT), and used as suggested by the supplier. A series of DNA fragment isolations (**A**, **B** and **C**) were performed using standard enzymatic reactions (Maniatis et al., 1982) and protocols for DNA recovery (Maxam and Gilbert, 1980; Weislander, 1979; Vogelstein and Gillespie, 1979). The isolated fragments were then assembled in different combinations (**D** and **E**) in reactions catalyzed by T4 DNA ligase. The pAVE1hGH (**F**) was constructed from pAVE1 and the *hGH* promoter-less gene fragment (**C**). Finally, pAVE2hGH22K (**G**) was assembled from the different DNA pieces as explained in section **c**. Vertically striped box, hPL-3 gene 5′ end; horizontally striped box, hPL-3 cDNA; stippled box, *hGH* 22-kDa hGH; open and hatched boxes, *hGH* gene 3′ end. Sizes are not drawn to scale. Ligated materials were ethanol-precipitated, resuspended in 10 mM Tris · HCl pH 7.5/0.1 mM EDTA and used to transform *E. coli* strain RRI using standard protocols (Maniatis et al., 1982). Rapid preparation of plasmid DNA from 3 ml overnight cultures was done by the alkaline method (Birnboim and Doly, 1979) with modifications consisting of phenol (saturated according to Maniatis et al., 1982) chloroform–isoamyl–alcohol (25 : 24 : 1) extraction of the clear lysate, replacing the Na · acetate in the high salt solution for K · acetate and finally reprecipitation with ammonium acetate and ethanol. Large-scale preparation of plasmid DNA for transfection studies was carried out by the clear-lysate method (Clewell and Helinski, 1971) followed by centrifugation through a CsCl-ethidium bromide gradient.

analysis and hGH RIA, respectively. Both the transiently expressed natural (from pAVE1hGH) or hybrid (from pAVE2hGH22K) *hGH* mRNAs were easily and specifically detected by hybridization with the ^{32}P-labeled *hPL* cDNA probe (data not shown). The RIA values of secreted *hGH* averaged 691 ng for the positive control gene and 921 ng for the hybrid cDNA gene, per 60-mm petri dish.

(d) Conclusions

The results presented here demonstrate the high versatility of our new pair of expression vectors. By capitalizing on the strength of the enhancer-promoter unit of hCMV, we have achieved efficient cell-culture expression of both the *hGH* split gene and its most abundant cDNA. We anticipate that the availability of these new vectors may improve the efficacy of transient expression experiments aimed at functionally dissecting mammalian structural gene sequences.

ACKNOWLEDGEMENTS

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CELL FUNCTION AND DISEASE

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THE HUMAN PLACENTAL LACTOGEN AND GROWTH HORMONE MULTI-GENE FAMILY

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INTRODUCTION

The central problem of molecular biology is the understanding of how the genetic information coded in the nucleic acid is expressed and, what are the mechanisms that regulate such expression. Much progess has been made in understanding gene regulation in prokaryotic systems, however, in eukaryotic organisms, the advances have been much slower and much more recent. This is closely related in part to the degree of evolutionary complexity of the eukaryotic cell.

The regulation of genetic expression is an essential characteristic of living cells. Of the total amount of genetic informacion possessed in all cells, only a small fraction is differentially transcribed within a certain time and space.

Studies on the expression of procaryotic genes showed that besides the region of DNA to be transcribed into RNA, sequences located both to the 5' side (operators, promoters, etc.) and 3' side (terminators) of this structural gene, are involved in regulating gene expression. In addition, according to the operon theory (1), for the regulation of the expression of bacterial genes, various elements are required such as: DNA-dependent RNA polymerase (the enzyme responsible for transcribing the gene); several types of protein factors associated with the enzyme (ρ rho, etc.) as well as metabolites (inducers, repressors, etc.) which interact with DNA to regulate bacterial gene expression (1).

Eukaryotic cells have approximately 10^3 times more DNA than a bacterium, multiple forms of DNA-dependent RNA polymerases (at least three types, each transcribing a different subset of genes) and compartmentalization of the processes of transcription (in the nucleus) and translation (in the cytoplasm). In addition to these differences, the discovery

in eucaryotes of split genes (2), and RNA processing, made it clear that regulation of eukaryotic gene expression is complex and can be exerted at a variety of different levels (3). The picture becomes even more complicated considering the organization of the eukaryotic genome into chromatin (4). In this regard, genes being expressed are said to be in an "active" chromatin configuration, although what determines this configuration is unclear.

THE PLACENTA AS AN EXPERIMENTAL MODEL

In evolutionary terms the placenta is recently acquired and a very efficient organ that functions to aid in the survival of the offspring and thus in the perpetuation of the species. Development of placental mammals allowed the mother to carry the unborn young with her while searching for food, thus protecting the fetus from predators. It also increased the area that could be covered during the searching process and facilitated migration. Thus, the placenta played a crucial role in the success of mammals colonizing the earth (5).

The placenta (6) is a remarkable organ in that is created from the same fertilized ovum that gives rise to the fetus but functions independently. The placenta exhibits unique characteristics that make it an excellent model for biological research. These properties include: a) its rapid growth and invasion of the maternal uterine tissue, b) the sudden stop of this invasion (by an unknown mechanism), c) the immunological processes that protect the placenta and the fetus from rejection, d) its hormonal regulation of pregnancy, etc.

In addition, the placenta is the organ with the highest rate of protein synthesis (7); and, since it develops and matures in less than 40 weeks, it constitutes an excellent system to study changes in gene expression during development and cell differentiation.

The placenta synthesizes a large variety of hormones (8). Probably the best characterized are chorionic gonadotropin (hCG) and placental lactogen (hPL, also know as chorionic somatomammotropin; HCS). While first trimester placental tissue is highly active in the synthesis of hCG with only low levels of hPL, in term placenta the situation is reversed (7). High levels of hPL (up to one gram per day) but low levels of hCG are produced by placenta at term.

In 1962, Josimovich and MacLaren (9) defined and characterized human placental lactogen as a polypeptide hormone present in extracts of human term placental and retroplacental blood that exhibited both potent lactogenic activity and an immunochemical reaction of partial identity with human growth hormone. hPL maternal blood levels are used to reflect the functional integrity of the placenta during pregnancy (10). hPL influences mammogenesis and lactogenesis as well as many aspects of the maternal intermediary metabolism directly related to the supply of nutrients for the metabolism of the fetus (11). However, the primary action of this hormone has not been defined.

The hPL molecule is a single-chain polypeptide of 191 aminoacids, produced in the syncytiotrophoblast layer (12). It contains two intramolecular disulfide bonds and no carbohydrate or lipid.

The hPL production is coupled to the development of the placenta, reaching its maximum towards the end of pregnancy. The great quantities in which this hormone is produced, makes this hormone ideal for research

and biochemical manipulations. For these reasons, we choose the placenta as the ideal organ to carry out the studies described in this paper.

RECOMBINANT DNA: A NEW AND POWERFUL TECHNOLOGY TO STUDY GENE STRUCTURE AND EXPRESSION

The birth of recombinant DNA technology in the early 1970's, marked the beginning of a new era in Molecular Biology. Recombinant DNA simply means the recombination in the test tube of different DNA molecules. This technology has provided us with very powerful tools and methods for the isolation, characterization and manipulation of gene sequences.

With the aid of this technology, it has been possible to begin the analysis of highly complex genomes of eukaryotic cells at the molecular level. Studies are being carried out to analyze the molecular structure and organization of genes in order to understand their function, regulation and origin.

The essential elements that constitute the group of recombinant DNA thechniques include:

I. Enzymes to modify DNA and RNA

Such as restriction enzymes, ligase, phosphatase, reverse transcriptase, DNA polymerase, polynucleotide kinase, etc. These proteins are employed to carry out the manipulation process of the DNA to be cloned. These genetic manipulations consist of specific cleavages along the DNA molecule or, modifications, covalent unions, radioactive labeling, etc.

II. Molecular Hybridizations

They can be performed using a liquid or solid support. This technique (13) consists of the detection, through the use of radioactive probes containing complementary sequences, of desired molecular species that are present in complex mixtures of DNA or RNA. These radioactive probes when denatured and later renatured in the presence of the mixture, form molecular hybrids with the desired single DNA chain or RNA. This coupling is stable due to the establishment of hydrogen bonds between the complementary nucleoside bases of the hybrid molecule.

III. Molecular vehicles

They include: plasmids (14), cosmids (15), lambda bacteriophage derivatives (16) and M13 bacteriophage derivatives (17). These are used to clone foreign DNA fragments (such as: human genes) and permit its propagation in bacteria; thus exploiting the following three qualities:

a. DNA fragments can autonomously replicate in host cells as they are inserted into vectors containing replication origins.

b. They can be separated from the bacterial nucleic acids and easily purified.

c. They contain DNA regions that are not essential for its propagation in bacteria. Foreign DNA inserted in these regions is replicated and propagated as if they were a normal component of the vector.

IV. Determination of the sequence and synthesis of DNA

This can be carried out either by enzymatic (18) or chemical (19) techniques. The sequencing methods for DNA (or including RNA) generate a great quantity of valuable information concerning the primary structure, organization, regulation, and evolution of the genes and proteins which they code. Thanks to these techniques, it is now much easier to determine the amino acid sequence of a protein through the sequence of its cloned messenger RNA (transformed to DNA through reverse transcription). On the other hand, it is possible to sequence a part of a protein whose gene we wish to isolate and characterize. With the information of the amino acid sequence and genetic code, it is possible to synthesize an oligonucleotide capable of serving as a probe to carry out molecular hybridizations. In this manner, the desired gene is isolated and characterized from a gene bank.

V. Gene library or bank

It is created (20) starting from either plasmids, bacteriophages, or cosmids; all containing either natural genes (gene bank) or DNA complementary (cDNA) to the messenger RNA population of a particular tissue or cell (cDNA bank). Basically, what is done with these banks is to take advantage of the classical bacterial models that have been so useful to elucidate the molecular basis of the regulation and expression in prokaryotics, to study the molecular genetics of higher organisms.

Briefly, to clone and isolate a gene, the following steps are carried out:

1. The genome under study is isolated
2. It is then fragmented by the use of restriction enzymes
3. The resulting DNA fragments are introduced in molecular vehicles, thus constructing gene banks.
4. A radioactive probe containing a complementary sequence to the gene portion that we propose to isolate, is used to identify the clone that contains the gene under study.
5. Sufficient quantities of the desired gene is purified.
6. The desired gene is characterized.

After carrying out these steps, the gene is used to perform the pertinent studies which help us understand the evolutionary history and gene functions in the living organism.

From the numerous studies carried out concerning the molecular structure and organization of the genes in eukaryotics, the term, split gene, has emerged (2). The majority of the genes in higher organisms are discontinuous. This means that the DNA which codes for the protein is interrupted by non-coding regions known as introns. These introns form part, as well as the code regions (or exons), of the primary transcript of the gene; but, are eventually eliminated to produce the mature mRNA which later is translated into a specific protein. In fig. 1, the molecular anatomy of the split gene model is outlined. Besides the introns and exons, some DNA regions or sequences are described. These DNA regions are also important to achieve a precise and efficient expression of these type of genes.

EXPERIMENTAL APPROACHES: THEIR STRATEGY AND INTERRELATIONSHIP

The experiments described in this section, were possible due to the development of Recombinant DNA techniques. Starting from the idea of exploiting the human placenta as the experimental model to study the mechanisms which regulate the specific genetic expression of the tissue, and using recombinant DNA techniques; our effort was focused towards the biogenesis of the most abundant protein in this organ, the Placental Lactogen hormone (hPL).

The particular objectives of our experiments were to identify the components and to elucidate the different steps involved in the genetic flow of information responsible for the synthesis and regulation of this polypeptide hormone.

CAT Box*: CAAT	Left Splice Junction: AG↓GTA
TATA Box: TATAAAA	Right Splice Junction: TXCAG↓
Capping Site: GTTGCTCCTXAC	Polyadenylation Signal: AATAAA

*position variable

Modified From: Lewin (1980)

= Untranslated region
= Flanking Regions
= Intron
= Exon

Fig. 1. Canonical protein-encoding mammalian gene. The figure shows the structural and regulatory sequences that characterize this type of genes.

In 1978, we started to investigate the genetic expression of hPL by trying to isolate and characterize the DNA dependent RNA polymerase type II of human placenta. This enzyme is responsible in carrying out the transcription of the genes that code for proteins. Because of this, it plays a key role in the functioning of the cell. However, this approach was somewhat premature since we first had to establish how to isolate and then biochemically and structurally characterize placental nuclei, which were to be our source of the enzyme. Once we accomplished this requisite (21) the isolation of the enzyme turned out to be a difficult project. Besides the inherent difficulties related with the technical procedures to be used, problems related with endogenous proteases and a lack of knowledge of the quaternary structure, were added on to our already existing problems. In spite of this, we were able to partially purify the enzyme and at least four of its subunits were detected (22). This was achieved through the use of electrophoresis in polyacrylamide gels with sodium dodecil sulphate (SDS).

With the coming of the DNA recombinant technology, the manner to attack the problem acquired a new focus. The molecular cloning of genes of higher organisms and the use of genetic engineering caused a revolution in the laboratories dedicated to the study of the gene expression and regulation. With a new plan in mind, adding these new techniques to the existing ones in our laboratory, we began to identify the elements that were involved in the different regulation levels of the biosynthesis of hPL (23). Briefly, we will mention our experimental strategy which can be used as a useful guide to present similar studies.

1) The in vitro synthesis of hPL and of its precursor form (pre-hPL) was studied (24).

2) The mRNA and pre-mRNA's for hPL were characterized (24).

3) The construction of a cDNA bank (DNAs which were copied from mRNA using the reverse transcriptase enzyme) was performed using terminal human placenta mRNAs (24).

4) The cDNA derived from the hPL mRNA was isolated (24).

5) This cloned cDNA was characterized, and sequenced (25).

6) The chromosomal localization of the hPL and hGH genes was carried out by in situ hybridization (26).

7) The number of genes for these two hormones was estimated (26).

8) The nuclear genes (27) for these hormones were isolated from a library of the human genome.

9) The cloned cDNA was hybridized against cloned hPL genes to form heteroduplexes (i.e. double stranded DNA molecules formed between one strand of cDNA and the complementary strand of the gene). The examination of these hybrid DNA molecules with the electron microscope, revealed the presence of four small introns in the hPL genes (24).

10) Two genes for hPL, two for hGH and one hPL-like gene were identified (23).

11) The expression of the genes for hPL were analyzed (25).

12) The hPL - like gene was sequenced showing similarities with the genes for hPL.

13) Elements involved in regulation of hPL transcription were identified.

The experimental projects and their results are described as follows:

MOLECULAR STRUCTURE

Molecular cloning of the mRNA for hPL

Using the same experimental model (the placenta) but with more powerful methods at hand, we proposed new and more ambitious questions:

Why is it that the placenta at term contains four to five times more translatable mRNA for hPL than first trimester placenta?. What were the

possible mechanisms involved in the specific regulation of the expression of the gene (or genes) for hPL?.

In an effort to respond to these questions, we decided to study the structure, abundance and origin of the mRNA for hPL.

Projects carried out by other investigators (28) indicated that the mRNA for hPL should contain approximately 900 nucleotides. In addition, a fragment of 550 base pairs corresponding to a portion of DNA complementary to the hPL mRNA (synthesized using reverse transcriptase and the DNA polymerase I of Escherichia coli) was already cloned in plasmid (29).

We isolated the total nucleic acids from term placenta. The high molecular weight RNAs were purified by the use of selective precipitation with 3M sodium acetate, pH 5.2. From these RNAs, the mRNA were selected through affinity chromatography in columns of oligo-dT-cellulose. This was achieved by exploiting the characteristic property of mRNA's possessing poly A "tails" in the 3' end.

The RNA messengers were translated in a cell-free system prepared from rabbit reticulocytes and mouse cell cultures. The synthesized proteins (which were radioactively labeled) were analyzed by the use of electrophoresis in polyacrylamide gels with SDS. By carrying out immunoprecipitation using anti-hPL serum two bands were observed: one that co-migrated with purified hPL and another more prominent, that most likely represented a pre-hPL (i.e. the immature form of hPL containing the signal peptide). The sum of these two bands represented approximately 15% of the total radioactively labeled protein.

When the RNA messengers were analyzed through electrophoresis in urea-acid-agarose gels, a prominent band of approximately 860 nucleotides was observed (24). A band of the same magnitude was observed when a recombinant plasmid, which contained the cDNA fragment of 550 nucleotides of hPL (30), was labeled with ^{32}P and hybridized against total RNAs fixed within filters. When the nuclear RNA was analyzed using this same method, four additional bands were observed of 990, 1200, 1460 and 1760 nucleotides. These most likely are the hPL mRNA precursors. The RNA messengers were also used to construct a cDNA bank.

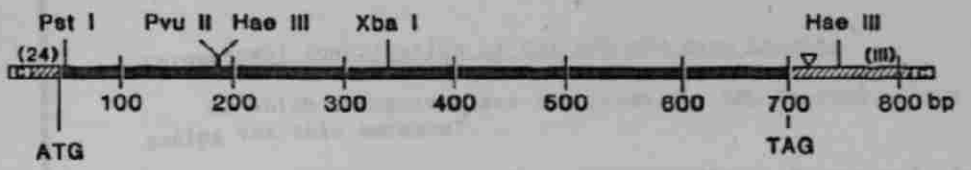
Approximately 5% of the recombinant clones, which constitute the bank, hybridized with sequences of hPL DNA. This indicated that the RNA messenger for hPL is certainly abundant in terminal placental tissue. One of the clones which scored positive in the hybridization, contained a cDNA of approximately 815 base pairs. This clone was isolated and characterized by the use of restriction enzymes forming the map seen in fig. 2.

cDNA molecules were hybridized with molecules of the hPL gene to construct what is known as a heteroduplex. The electronic microscope analysis of the heteroduplex reveled the presence, in the hPL gene, of four small introns or intervenient sequences (fig. 3) which explains the presence and sizes of the four precursors for the hPL mRNA present in the nuclear RNA.

The importance of these results is that they established for the first time the following:

1. The complete map of the recognition sites of the restriction enzymes present in the complementary DNA of the mRNA for hPL.

2. The existence of precursors (pre-mRNA) of the mRNA for hPL.



coding region

untranslated regions

dC-tail

tetramer insertion

Fig. 2. Map and organization of the hPL cDNA insert of phPL815. It contains the 651 nucleotides (nuc.) coding for the 26 aminoacids of the signal peptide and 191 aminoacids of the mature hPL hormone. In addition, the insert also includes 24 nuc. of the 5' unstranslated region and 111 nuc. of the 3' untranslated region.

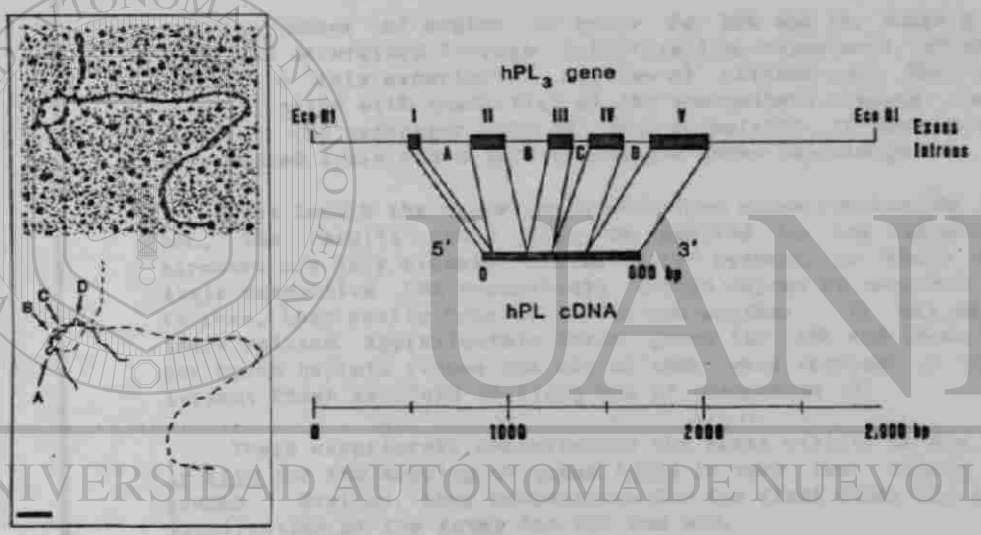
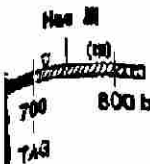


Fig. 3. Molecular structure of the human placental lactogen genes. The information obtained from the analysis of 16 heteroduplexes, as the one of the electron micrograph at the left, was used to determine the molecular structure of the human placental lactogen genes (represented by hPL_3). The heteroduplexes were formed by hibridization of plasmid DNA (phPL815, dashed line) containing the cDNA to hPL mRNA, with the DNA containing the hPL_3 gene (solid line). Four small intervening sequences are detected in the heteroduplex region at positions indicated by arrows labeled A-D. Magnification is indicated by bar length of 0.1 µm in lower left corner.



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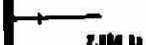

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3. The presence of four introns in the gene for hPL.

Chromosomal localization of the hPL-hGH gene complex

On which chromosome are the genes for hPL located on and how many are coding for this hormone?.

To directly visualize the genes in their chromosomal location(s) an experiment involving hybridization of DNA in metaphase chromosomes with a radioactive labeled probe (^3H) was carried out. The probe consisted of a portion of complementary DNA to the messenger RNA for the (hPL) which was cloned in the molecular vehicle known as pBR322 (14).

The molecular hybridization was carried out in the presence of dextran sulphate 10%, which accelerates by ten- fold the velocity of the process of hybridization. Another important factor which decisively contributed to our success in the chromosomal localization of genes with very few copies per genome, was the presence of the DNA chains of the vector covalently linked to the insert. These single chain DNAs hanging from the site of hybridization where the insert is hybridized served as an anchor for multiple hybridizations between complementary chains of the denatured plasmid, resulting in an increased accumulation of specific radioactivity.

The number of copies of genes for hPL and for human growth hormone (hGH) was determined through hybridization experiment on nitrocellulose filter. In this experiment, samples of chicken cell DNA were tested in parallel mixed with quantities of the recombinant plasmid (containing the cDNA for the messenger RNA of hPL) calculated in such a way that they represented 1,2,4 and 6 gene copies per human haploid genome.

Even though the experiments described above involve DNA sequences for hPL, the results could also be applied to the hGH genes. These two hormones are very closely related with respect to their evolution and their respective DNA demonstrate a high degree of sequence similary; due to this, they easily hybridize with one another. It was discovered that there existed approximately three genes for hPL and three genes for hGH per human haploid genome and all of them were located in the chromosomic segment known as 17q22-24 (long arm of chromosome 17)

These experiments demonstrated the great utility of the hybridization in situ for the mapping of genes found in very few copies in the human genome. Besides, they demonstrate for the first time, the subchromosomal localization of the genes for hPL and hGH.

Isolation and characterization of the members of the multi-gene family

Having verified the number, the chromosome localization, the molecular anatomy of the hPL and hGH genes, and counting with the complete messenger RNA for hPL made from cDNA and finally cloned; we proceeded to isolate clones from the human gene bank (27). These clones contained complementary sequences to a portion of the cDNA for the mRNA of hPL cloned (30) into pBR322. This recombinant plasmid, proportioned by Dr. Peter Seeburg, is known as pBR322-HCS, (HCS stands for Human Somatomammotropin, name also given for hPL).

Kidd took DNA from the recombinant plasmid, labeled it with ^{32}P, denaturalized it, and immediately hybridized it against phage recombinant DNAs originating from the gene bank. From a total of 900,000 plaques of

phages analyzed, the seven that resulted positive, were grown and their DNAs purified. The DNA obtained from each recombinant phage, was characterized using restriction enzymes and hybridized against the hPL cDNA to locate regions containing hPL or hGH genes. Seven different genes were identified: hPL_1, hPL_2, hPL_3, hPL_4, hGH_1, hGH_2 and hGH_3.

In at least two phages, we were able to verify the connection between a hPL and a hGH gene. In addition, in another phage, we confirmed the linkage between two hPL genes. This indicated that probably all the genes were related with each other; thus suggesting, that all of these genes evolved from a common ancestor and that they originated by mechanisms of gene duplication and diversion. In more recent studies we established that hPL_1 and hPL_4 were the same gene, reducing to three the number of genes for hPL (hPL_1, hPL_4 and hPL_2). Also, the existance of the hGH_2 gene was not confirmed.

The Gene Structure

The molecular structures of the genes were obtained by four different methods. These were: 1) hybridizations on nitrocellulose filters (13) of the labeled cDNA (probe), against DNA from the fragmented genes obtained by the use of restriction enzymes. 2) comparing maps that contained various restriction enzyme cutting positions carried out for every gene, 3) confirming information obtained by determining the nucleotide sequence of the regions that flank the initiation and termination points of the genes, as well as, the borders between the exons and introns. 4) and finally, obtaining formation from the literature concerning the sequences of the cDNA and the gene for growth hormone, described by other groups of investigators (31,32). Four of the genes showed a very high degree of nucleotide sequence similarity with each other, as well as quite similar restriction enzyme maps. However, by: 1) the presence of repeated sequences adjacently to the 3' end of the gene, 2) the length of the fragments flanked by EcoRI sites and 3) the presence of characteristic restriction sites, it was possible to distinguish and identify each of these genes fig. 4). For example, while the two hGH genes are contained within Eco RI fragments of 2.6 kilobases (kb), both contain repetitive sequences near their 3' end region. Furthermore, both have unique sites for BglII. Even though these genes possess these similarities, one of them (hGH_1) has only one BamHI site in its fourth intron. As another example, we can mention the following. Two of the genes for hPL are contained within fragments Eco RI of 2.9 kb and possess unique sites for both XbaI and BamHI. However, they can be distinguished from each other by the presence of one (hPL_3) or two (hPL_4) FvuII sites. As a final example, the fifth characterized gene (hPL_2) is contained in a EcoRI fragment of 8.5 kb, which can be cut with XbaI, liberating a fragment of 3.5 kb containing the gene. In addition, this gene is characterized by the absence of XbaI and BamHI sites.

Molecular Anatomy of the Multi-gene Complex

The results obtained through the in situ hybridization experiments indicated that the genes were grouped within the region between the bands q22 and q24 of chromosome 17. Due to the quantity of DNA contained within this chromosomal region (several millions of base pairs), little could we deduce concerning the organization of these genes. Thus various questions arise: how close are these genes with respect to each other? What is the spatial relationship that the hGH genes have with respect to one another and with the hPL genes? Is each gene transcribed from the same DNA chain thus maintaining the same sense of transcript direction; or, do members exist that are transcribed from the opposite chain?

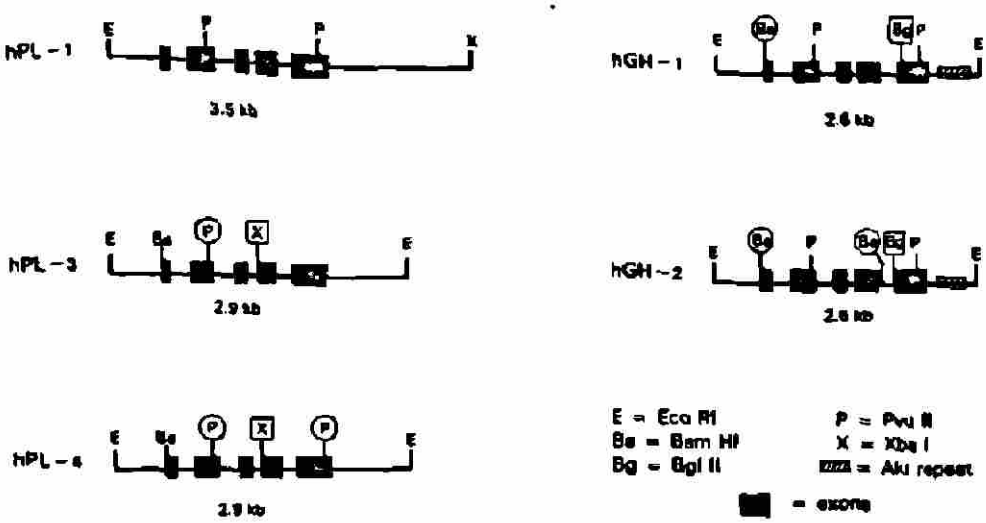

Fig. 4. The members of the hPL-hGH multi-gene family. Those enzymes useful to destinguish the different genes are in circles or boxes. hGH-1 is the same as hGH_N, while hGH-2 is also known as hGHv.

To solve these inquiries not only the data obtained from the isolated recombinant phages were analyzed; but, also we used information from other laboratories who were also working on the same theme. The data obtained from the molecular genetic analysis of patients who presented a congenital absence of hGH or hPL was also very useful to us. For example, a patient who presented with an absence of hPL (see Fig. 5), was shown to possess a deletion of approximately 35 kb with the loss of the genes hPL_1, hGH_1 and hPL_2. It was concluded that at least these three genes were quite close to each other. Having found recombinant phages containing two genes which were always one of the hPL type and the other of the hGH type, was particulary valuable in the establishment of the molecular anatomy of the genetic complex for hPL and hGH. This indicated that not only are the genes close to each other but, that they are also interwined with respect to their spatial arrangement along the chromosome. In fig. 5, the map proposed for the genetic complex is illustrated. This map was later confirmed by isolating cosmid clones containing all the gene members of the hGH-hPL gene complex (33).

GENETIC EXPRESSION

hPL gene expression

What is the reason for the multiplicity of the hPL genes? What might be the function of these genes? Might it be that all are transcriptionally active in the term placenta or, are some of them pseudogenes? To answer these questions the following experiments were designed.

Fifteen recombinant plasmids were selected from a cDNA bank of term placenta RNA, that tested positive when they were hybridized to detect complementary sequences to hPL cDNA. Having knowledge of the map indicating the characteristic cleavage sites for the restriction enzymes of each hPL gene (see fig. 4); we proceeded to cut the DNAs of these 15 plasmids

with the restriction enzymes. This was performed with the objective of finding cDNAs corresponding to hPL transcripts of the hPL_1, hPL_2 and hPL_3 genes. By using this method, these genes could be distinguished by the absence or presence of certain sites of the diagnostic restriction enzymes for each gene. We were able to detect plasmids with insert characteristic of hPL_1 and hPL_2 but not however for the characteristics expected of a transcript of hPL_3. In this manner, we were able to establish for the first time, that hPL_1 and hPL_2 were transcriptionally active in term placenta; and that possibly, hPL_3 was a pseudogene or that it is expressed in other stages of the placental development. Both cDNAs were characterized and sequenced. Their representation in the term placenta, determined by three different experiments, was estimated to be in the proportion of two to three for the RNA messengers of hPL_1 and hPL_2 respectively.

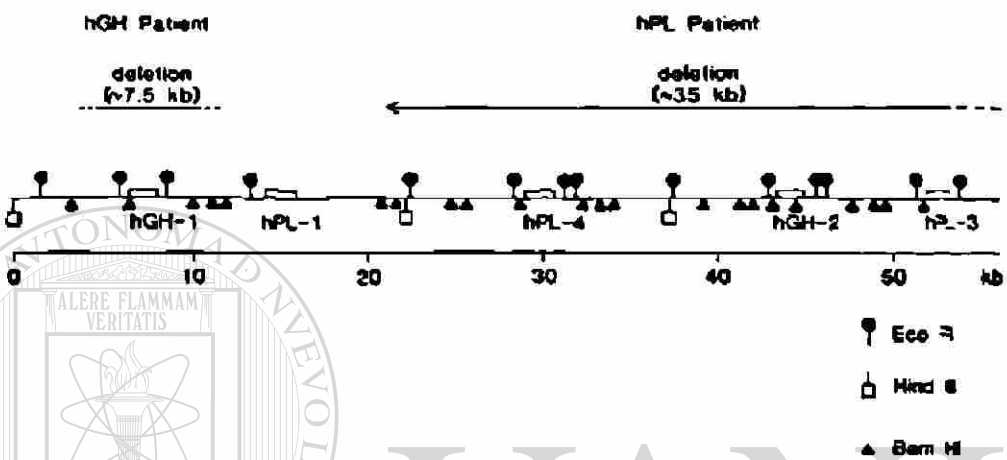

Fig. 5. Linkage map of the hPL-hGH multi-gene cluster. This map was constructed using information from restriction enzyme analysis of recombinant phages containing hPL and hGH genes, and the analysis of information from a molecular study of DNA from a patient with antenatal deficiency of hPL (see ref.46). The areas believed to be deleted in this patient and in a patient with familial isolated growth hormone deficiency are indicated above the map.

Finally, comparing the sequences of both cDNAs, 10 differences in the nucleotide positions were detected; although only one of them, caused a change in the amino acid sequence. This change is located in the signal peptide, which means that when this protein is processed before secretion, the mature protein coded by each of the two different genes is identical.

DNA sequences involved in regulation of the hPL gene.

Most cellular and viral genes have been shown to contain cis-acting sequences which regulate transcription of the gene. Some of these sequence include the TATA box and CAAT box located in the upstream promoter regions. Also hormone receptor sites, enhancers, and sequences which bind general or tissue specific factors can alter gene expression. These regulatory regions may be found upstream, downstream or within a gene.

We surveyed the entire hPL-hGH gene cluster for the presence of transcriptional enhancers (34). The gene cluster was digested with EcoRI and each restriction fragment was tested, using a transformation assay, for enhancer activity in hPL producing choriocarcinoma cells. The only enhancer detected was located 2 kb 3' to the hPL_1 gene (fig. 6).

A vector construct (fig. 7) containing the hPL enhancer linked to the reporter gene chloramphenicol acetyl transferase (CAT) under the control of the SV40 promoter was used in transfection studies to further characterize the hPL enhancer. These studies showed the enhancer was tissue specific as it was active only in cells which produce hPL. Transfection studies using choriocarcinoma cell lines have sublocalized the hPL enhancer to a 730 bp AccI-AvaI restriction fragment.

Deletion analysis of the enhancer suggests that multiple sequences throughout the 730 bp enhancer are necessary for enhancer activity. One region may be more important than others as over 60% of enhancer activity was found within the 5' most 210 base pares (bp). Protein binding studies suggest another 265 bp region (region II, fig. 6) may also be important. This region specifically binds protein found only in placental nuclear extracts. Regions I and III bind nuclear proteins common to both cells which produce hPL and cells which do not produce hPL.

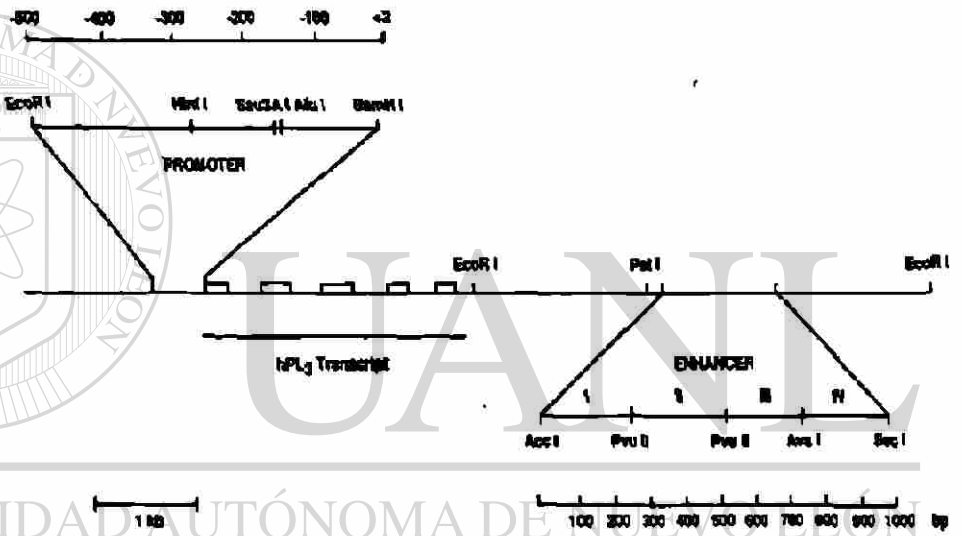

Fig. 6. hPL gene and regulatory sequences. Restriction enzyme digestion maps of both the promoter and enhancer regions are shown. Boxes correspond to hPL_1 exons. Enhancer restriction fragments tested for nuclear protein - DNA interactions are denoted as I, II, III and IV.

DNA sequences extending 500 bp upstream of the hPL and hGH genes are 95% homologous. When these 500 bp regions were inserted 5' to the CAT gene they were each shown to have low promoter activity. However, when the hPL enhancer was linked to either the hPL or hGH promoter, a marked difference in transcription activity was noted. Transcription activity of the hPL-promoter hPL-enhancer pair was 10-fold higher compared to that of the promoter alone, and 5 fold higher than the hGH-promoter hPL-enhancer

combination. This suggests that the hPL enhancer may act preferentially on the hPL promoter.

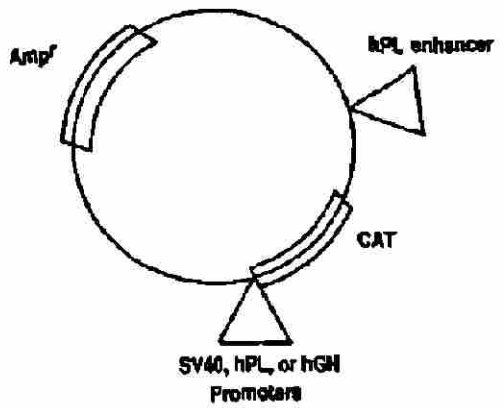

Fig. 7. CAT vector for hPL promoter and enhancer studies. Plasmid vector containing the promoter less bacterial gene for chloramphenicol acetyl transferase (CAT). Promoter sequences were inserted 5' to CAT and enhancer sequences added 3' to CAT.

The hPL promoter was analyzed to determine sequences that are important for regulating the transcription of hPL. Deletion mutants were created by digesting the 500 bp hPL promoter with various restriction enzymes. The resulting DNA fragments all contained the same 3' end but varied in length of 5' sequence (fig. 8). The deletion fragments were ligated 5' to the CAT gene in a vector that also contained the hPL enhancer to increase activity (see fig. 7). The plasmids were transfected into placental choriocarcinoma cells and the level of CAT activity assayed to determine the transcriptional strength of each promoter mutant. Maximal activity was seen when the promoter contained 390 bp of DNA and decreasing the size of the promoter to 152 bp did not lower this high activity. Initial experiments showed lower activity when the promoter fragments contained 2300 bp or 500 bp of upstream DNA sequence. A pronounced decrease (7-10 fold) in CAT activity was seen when the promoter was reduced to 129 bp. This low activity was also seen when the sequences between -152 bp and -129 bp were removed from the 390 bp fragment (390 SA). A new 142 bp clone was prepared and it had activity similar to the 152 bp clone indicating that an important region for transcription is contained between the DNA sequences -142 and -129 bp. This sequence may be important for binding trans-acting factors which stimulate the transcription of the hPL gene.

The hPL genes contain sequences both 5' and 3' to the genes that are important for its transcriptional control. Further study of these regions may elucidate their mechanism(s) of action in the regulation of hPL expression.

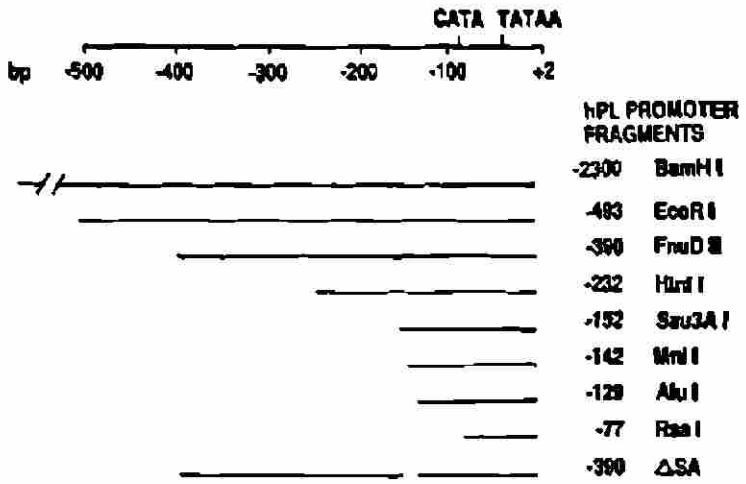

Fig. 6. Deletion mutants of the hPL promoter. Deletion mutants of the hPL promoter were created by digesting the DNA with various restriction enzymes. The fragments (solid line) all had identical 3' ends but decreasing amounts of 5' DNA.

Is hPL$_4$ a pseudogene?

What was the nature of the hPL$_4$ gene? What type of protein was coded in its nucleotide sequence? Was this gene, whose transcriptional products we could not find in term placenta, potentially functional? or did it contain mutations that rendered it as a pseudogene? To obtain an insight into these uncertainties, we decided to dissect the hPL$_4$ gene.

A 3.5 kb EcoRI - XbaI DNA fragment containing the hPL$_4$ gene was digested with various restriction enzymes. Aliquots of each of these digestions were ligated to M13 vectors that had already been cut with the appropriate restriction enzymes to generate compatible ends for cloning. Recombinants for each of the different fragments spanning the gene were identified and their single stranded DNAs were purified. The method of Sanger (18) was used to determine the sequence of the inserts in these M13 recombinants. The analysis of the hPL$_4$ sequence indicated that the characteristics of this gene are very similar to those of the other members of hPL-hGH gene complex. However, in spite of these similarities this gene possesses unique features. Both, the common and unique characteristics of the hPL$_4$ gene with regard to the other hPL genes are listed below.

1. It contains five exons of identical length to those in the other hPL and hGH genes.

2. It contains normal TATA (-30) and CAT (-84) boxes.

3. It contains a normal polyadenylation signal which is followed by a characteristic truncated Alu element located 100 nucleotides further downstream.

4. The protein that it encodes differs in 16 amino acid positions, as compared to the mature hPL protein coded by the hPL_4 and hPL_3.

5. The hPL_1 gene contains a G to A transition at the 5' splice consensus site of the second intron; thus, preventing pre-mRNA processing at this site and classifying hPL_1 as a pseudogene candidate.

When we searched for the presence of hPL_1 gene cDNA clones in a human term placenta cDNA library, we did not detect them (25). The search for transcripts of this gene by other group of investigators (35), who used hPL_1 gene specific oligonucleotides, was also unsuccessful. This evidence along with the presence of the point mutation at 5' splice site of the second intron of hPL_1, that in other genes (36) has been proven to be a cause of gene inactivity; strongly suggested that this gene was a pseudogene.

More direct experimental evidence was required to reach a definitive conclusion regarding hPL_1 gene expression. We decided to test in a transient expression experiment, if the splice site point mutation in hPL_1 rendered it unable to generate a mature mRNA. We rationalized that adequate controls for this experiment should be to introduce the mutation present in hPL_1 into a normal hPL gene and see if it now becomes defective in the production of mature mRNA, and to revert to wild type the mutation in hPL_1 to see if it now becomes active. The assumption behind these experiments was that the point mutation was the only cause of hPL-1 apparent gene inactivity. Next, we went on testing this assumption.

Comparisons of the nucleotide sequences around the region of the 5' splice donor site, contained in the active hPL-3 gene and the putative hPL-1 pseudogene, made it clear that we could easily exchange the mutated area between these two genes. We found PvuII and SacI sites located 30 base pairs upstream and 66 base pairs downstream of the mutation site, respectively. The sequences in this restriction fragment, of approximately 120 bp, differs among the two genes in only four nucleotide positions. In addition to the single point mutation in the 5' donor splice site, there were three other nucleotides sites within this region where these genes differ. The additional changes, however, are located inside the intron at 6,27 and 64 bp downstream from the exon-intron border, positions that could be taken as of little importance for the pre-mRNA processing.

We carried out the shuffling of this restriction fragment among hPL_1 and hPL_3 genes. To our advantage, the differences between these two genes inside the second intron sequences, were associated with an AluI site. Thus, the successful exchange of the PvuII-SacI fragment could be monitored by AluI digestions of the recombinant hybrid genes.

Furthermore, to leave no doubt of the identity of the recombined genes, we determined their nucleotide sequence in the area around the exchange site. In this manner we were sure we had our recombined and wild type genes to test our hypothesis.

We next constructed a new plasmid vector derived from pCMVCat (a plasmid containing cytomegalovirus enhancer-promoter control sequences fused to chloramphenicol acetyl transferase structural gene). This construction was shown previously to be a powerful expression system (37). Our new vector called pAVE-1 contains: pBR322 (14) sequences from the EcoRI site to the AccI site; the enhancer-promoter sequences of cytomegalovirus (37); and the polylinker region of pUC18 (38). We then

subcloned into pAVE-1 the structural regions of our test genes and were ready to assay their expression (fig. 9).

To determine the expression of our hybrid genes, we choose to introduce them into cultured cells (transient expression-assay) by the technique of calcium-phosphate-DNA coprecipitation (39). The efficiency of transfection was evaluated by using the CAT assay carried out on a fraction of the cultured cells cotransfected with both the test gene and pCMVCat.

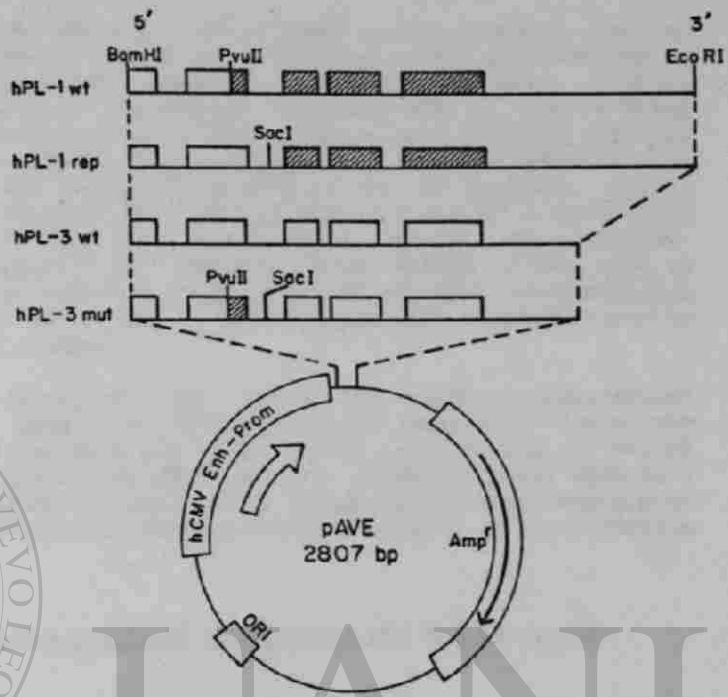

Fig. 9. Subcloning of hPL genes into the expression vector. The hPL$_1$ genes were directly cloned in pAVE-1 from their Bam HI site at beginning of the first exon to the Eco RI site at the 3' end of the gene. A different strategy was used for the hPL-1 genes since they lack the Bam HI site. To clone them, we replaced the 5' end of these genes with that of the hPL-3 gene, from the Bam HI site to the Pvu II in the second exon.

We isolated total RNA from the remaining transfected cells by the method of guanidinium isothyocianate-lithium chloride (40). Then we glyoxalated the RNA and separated it in an agarose gel. We transferred the RNA to a nitrocellulose membrane and hybridized it with an oligolabeled hPL cDNA probe. The preliminary results from this type of analysis indicated that the wild-type hPL-1 gene is not capable of producing a mature mRNA and that when its mutated splice donor site is substituted with a normal one this gene deffect is reversed. On the other hand, the wild-type hPL-3 gene produces good levels of hPL mRNA and which are

drastically decresed when this gene harbors the mutation present in hPL-1 gene.

Therefore, we concluded that hPL-1 is a pseudogene because, even when it has a high similarity to the *hPL active genes, does not produce* a mature mRNA.

hGH gene expression

Even though the 22 kilodaltons form of human growth hormone, the one which normally circulates in the blood, is coded by the gene located in the 5' region of the multi-gene complex (the hGH_1, or hGH_n, n being "normal"). The expression of the second hGH gene, hGH_2 or hGH_v (v being "variant"), has been detected using an expression vector derived from the monkey simian virus 40 (SV40). When cells in culture are infected with this recombinant virus the transcription of gene can be observed; and as result, there is a production of a hormone that differs in 13 amino acid positions with respect to hGH_1 (41). More recently, by the use of synthetic oligonucleotides specific for the hGH_2 gene, levels of hGH_2 mRNA at least four orders of magnitude lower than those of hPL mRNA, were detected in term placenta (42). Up to now, it is not known what possible function may have this hGHv gene product.

The complications do not end here since the primary transcription product of the hGH_1 gene, is regulated in its processing. Due to the presence of two alternative splicing sites in the elimination of one of its intron (43); the additional mRNA that is produced, gives rise to a variant of the normal growth hormone (of 20 kilodaltons), which represents approximately 10% of the growth hormone activity present in the pituitary gland.

Expression and functional analysis of transfected hGH and hPL genes

hGH and hPL are the two well characterized products of the hGH and hPL multigene complex. They share 85% of their aminoacids. hGH is unique among the animal growth hormones in that it possesses prolactin-like activity. hPL also shows prolactin-like activity and in spite of its sequence similarity to hGH, it is virtually inactive as GH. The structural similarities and functional differences of these hormones offers a good opportunity to study the evolution of functional domains in proteins.

We are interested in studying the possible functions of the less characterized members of this multigene complex. While the major product of the hGH_n gene (the 22 kd form of hGH) and the identical mature protein encoded by both hPL_1 and hPL_2 genes are easily obtained from pituitary gland and placenta, respectively, this is not true for the putative protein products of the remaining gene members. The proteins encoded by the hGHv and the hPL_3 genes, and any other minor products generated through differential splicing from any of the gene members can only be studied by recombinant DNA approaches.

As a neccesary step to pursue our objetive we decided to stablish an efficient expression system based in the transfection of genes into cultured cells. Starting with the *hGH$_n$ gene*, we have constructed new hybrid genes (Fig. 10) by joining the structural region of this gene with various types of transcriptional control elements. The novel joints plasmids harbor the following promoter and/or enhancer elements: the mouse metallothionein 1 promoter (pMThGH), the mouse metalothionein promoter together with the SV40 enhancer (pNUThGH), the promoter-enhancer of the

immediate early gene of human cytomegalovirus (pCMVhGH), as well as the natural promoter of the gene in conjunction with the SV40 enhancer (pSVgpthGH). As a result, we have achieved a good level of expression of this structural gene by transfection into COS-7 cells. Both Northern blotting and radioimmunoassay were performed to evaluate the strenght of these transcriptional control elements and hormone secretion, respectively. The best secretion of hGH was achieved using the pNUThGH plasmid. The potency of the remaining novel joints can be ordered, going from the strongest to the weakest as follows: enhancer-promoter of CMV, natural promoter in combination with the SV40 enhancer, and finally, we obtained the lowest secretion of hGH into the medium when using alone the promoter of MT-I.

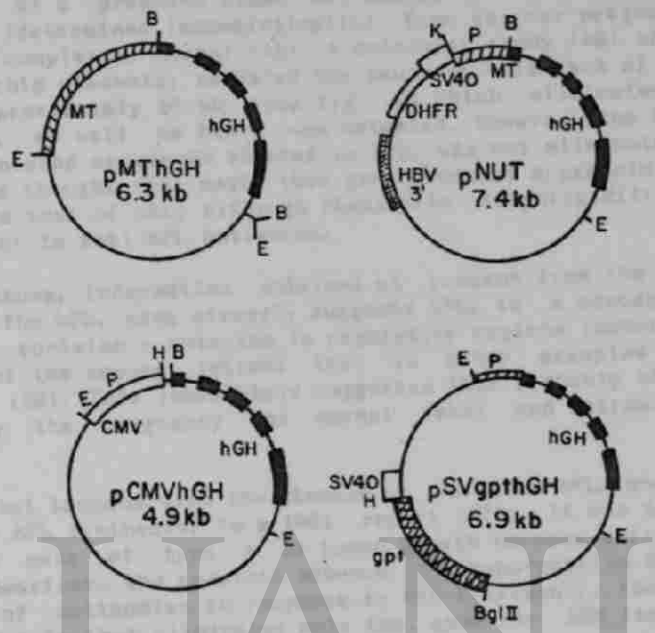

Fig. 10. Structure of hGH expression vectors. With the exception of pSVgpthGH, which contains the entire hGH gene (including its natural promoter), all the other vectors shown here were constructed by fusing the hGH gene structural region (BamHI site at the beginning of the first exon to EcoRI site at the 3' end) to various transcriptional control elements. In pMThGH expression of hGH promoter-less gene is directed by the mouse metalothionein promoter. In pNUT act both SV40 enhancer sequences and mouse metalothionein promoter. Finally, in pCMVhGH the transcriptional control element is the enhancer-promoter of the inmediate early gene of human cytomegalovirus.

Our results confirm those obtained by Pavlakis et al (41). Using SV40 vectors carrying the structural region of the hGH$_N$ and hGHv genes, they

showed that monkey kidney cells were able to process hGH prehormone and secret the mature hormone into the culture medium. The amount of hGH secreted in our transient expression experiments was comparable to that secreted by Vero cells lines permanently transfected with a plasmid carrying an SV40-hGH hybrid gene (44). Therefore, we are now ready to produce enough quantities of the other protein products encoded by the hPL-hGH gene family members, to start an analysis of their possible functions.

MOLECULAR BASIS OF THE DEFICIENCIES OF hGH AND hPL

In 1982, a case of a pregnant woman was described who presented a total absence of hPL (determined immunologically). Even so, her pregnancy, birth and child were completely normal (45). A molecular study (46) of the hPL-hGH complex in this placenta, revealed the cause of this lack of hPL. A DNA deletion of approximately 35 kb (see fig. 5) which eliminated the hPL_1 and hPL_2 genes, as well as hGHv, was detected. However, the fifth gene (hPL_f) which contains sequences related to hPL_1 was not eliminated in the deletion. It was thought that maybe this gene produced a protein with activities similar to that of hPL; although chemically slightly different since it did not react to anti-hPL antiserum.

As described above, information obtained at present from the molecular dissection of the hPL_1 gene strongly suggests hPL_1 is a pseudogene. This is because it contains a mutation in regulatory regions (donor site for the processing of the second intron) that in other examples would inactivate the gene (36). This immediately suggested that probably hPL may not be necessary for the pregnancy and normal fetal and extrauterine growth.

Contrary to what happens with the absence of the genes hPL_1 and hPL_2 responsible for the hPL synthesis, in a 1981 report (47), it was demonstrated that in a case of type A of human growth hormone deficiency (characterized by dwarfism, the complete absence of immunoreactive hGH and by the generation of antibodies in response to hGH treatment), the cause was a deletion of 7.5 Kb that eliminates only the gene for hGH (see fig. 5).

MOLECULAR EVOLUTION

The sequence analysis of the genes has given us valuable information to allow us to try to reconstruct the evolutionary process of the structure of the hGH-hPL complex. In 1971, Niall et al. (48) noticed the presence of similar aminoacid regions in the growth, placental lactogen and prolactin hormones. This observation indicated that these hormones were probably related to each other with respect to their evolution. This was also suggested by their physiological activities, since the three show overlapping activities in different degrees. These observations lead to the hypothesis the genes for these hormones constitute a family, from an evolutionary point of view, having originated from a common ancestor. This common ancestor, which is believed to be similar to prolactin (since it exists in all vertebrates while the other two hormones evolved later in higher vertebrates and placental mammals), underwent a gene duplication and subsequent divergence of the products. This implies that there existed at least two events of genetic duplication during the evolution of these hormones (fig. 11). The first established the branches of prolactin and of growth hormone. The second, most likely occurred in the growth hormone branch, giving rise to the placental lactogen hormones, the youngest member of this family.

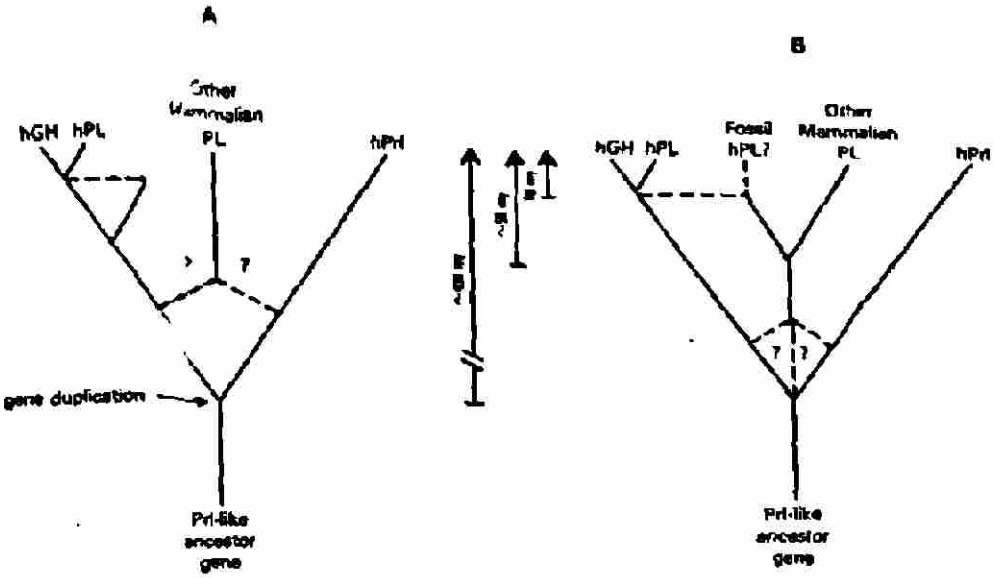

Fig. 11. Possible pathway for evolution of hPL in mammalians. Two alternative hypothesis are presented. In both of them a gene conversion mechanisms has rendered the aminoacid sequences in hPL and hGH very similar (85%).

Using taxonomic, amino acid, and nucleotide sequence comparisons, it has been estimated that the first genetic duplication occurred approximately 400 million years ago. This estimation goes back to the times when the evolutionary divergence of fish and tetrapods occurred; since the pituitary glands of amphibians, reptiles and birds contain different molecules similar to prolactin and growth hormone.

Estimations of the evolutionary origin of the hPL gene, using two different criteria, give conflicting results. The necessity of placental lactogen accompanied the origin of the principal orders of placental mammals, approximately 75 million years ago. On the other hand, the branching of the primates occurred about 60 million years ago, considered to be the approximate time when the hPL appeared. However, the analysis of the amino acid and nucleotide sequence suggest that the appearance of an hPL gene by duplication from its ancestor hGH gene occurred approximately 10 million years ago. To resolve this paradox, it has been postulated (49) that gene conversion must have occurred (a non-reciprocal, recombinant mechanism where a gene serves as a mold to correct mutations in another gene). The fact that the genes for these two hormones are contiguous on the same chromosome, could have facilitated this gene conversion; furthermore this event must have happened about 10 million years ago (see fig. 11).

Finally, the observations of the internal homologies of these three hormones, suggest that the common ancestral gene could have resulted from a repetitive duplication process of a gene that coded for a primordial peptide of approximately 20 amino acids (48).

PERSPECTIVES

The results presented here allow us to elucidate, at a molecular level, the route of the genetic flow of information for the Placental Lactogen hormone. Since its starting point in the nucleus of human placental cells and through the different levels of expression, this genetic information is a product of the structure, function, and evolution of this hormone.

Now we know the structure of the two active genes responsible for the production of the hPL. They are relatively small genes (the sequences present in mRNA are approximately 800 bp distributed in five exons interrupted by four small introns). These two genes are within a cluster that includes two genes for the growth hormone and a fifth gene that contains sequences that identify it as similar to the hPL genes. These genes are distributed in approximately 50,000 bp of DNA in the q22-24 region of the human chromosome 17.

In spite of the advances achieved, there is still much to be investigated concerning the expression and regulation of this genetic complex. We know nothing about the expression of the hPL genes during pregnancy nor in trophoblast pathologies. We are just begining to understand the mechanisms (in spite of the proximity and very high similarity of its genes) by which the cell manages to control the specific expression of the genes such that hPL is produced in the placenta, while hGH be produced in the pituitary gland. The causes of sudden increase of hPL synthesis during the second triaester, as well as the regulation of the synthesis of hPL are still unknown. We do not know if hPL is involved in the mechanisms of initiation of labor during birth, nor if it has any role in the immunological concealment that the placenta and fetus possess to avoid being rejected by the maternal immune system.

It is necessary to perform more definitive studies in relation to the expression of the hGHv gene, to better understand physiological role that its gene products might have.

It is clear that the studies described in this assay, allow a better understanding of the molecular basis that control biological processes so important such as cellular differentiation and development. A better understanding of these normal processes would help clarify the causes of genetic and other type of ailments, the aging process, and the origins of cancer.

Certainly, this is a good model to carry out studies related to genetic regulation and we hope to continue learning from it.

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Functional expression of human pituitary growth hormone complementary DNAs.

(Recombinant DNA, hGH DNAc; differential splicing; transient expression; eukaryotic expression vector; adipogenic activity)

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Abbreviations: bp, base pair(s); hGH, human growth hormone; hPL, human placental lactogen; kb, kilobase pair(s); RIA, radioimmunoassay; met hGH^{phe}, recombinant hGH.

SUMMARY

We have isolated, cloned and assayed the functional expression of pituitary cDNAs for human growth hormone (hGH). A selective cDNA cloning strategy was used to preferentially obtain both hGH 22K and hGH 20K cDNAs. These were used to construct minigenes which were subcloned into the eukaryotic expression vector pAVE2 [Ramírez-Solís et al., Gene 87 (1990) 291-294]. Transfection assays in COS-7 cells of both minigenes proved they are similarly efficient in generating mature RNAs, and allowed the detection and visualization of the secreted hGH-22K and hGH-20K polypeptides. Finally, the hGHs transiently expressed and secreted into the culture medium by the transfected COS-7 cells were able to specifically promote differentiation of preadipocytes 3T3-F442A to adipose cells.

INTRODUCTION

The growth hormone normal (hGH-N) gene is the only member of the five gene human growth (hGH)-placental lactogen (hPL) hormone multigene family that is expressed in the pituitary gland [1]. In spite of its high (~85%) aminoacid sequence similarity with hPL; hGH has, as one of its distinctive functional features not shared by hPL, the capacity to induce differentiation of adipocytes [2].

Differential splicing of the hGH-N gene primary transcript generates two different mRNAs. The translation product of one of these mRNAs is the well characterized 22-KDa form of hGH (hGH-22K). The other mRNA contains a 45bp internal deletion and encodes the 20-kDa form of hGH (hGH-20K) [3-4]. The 20K hGH mRNA and its corresponding hormone are present at very low abundance in the pituitary gland.

In vitro mutagenesis [5] and molecular cloning experiments [4] have led to the isolation of the 20K hGH cDNA. Biological activities of the 20K-hGH have been found to be equipotent to 22K-hGH in various growth-promoting assays such as tibial tests, body weight gain tests and in somatomedin synthesis [6-8]. However there is controversy related with their effects on carbohydrate and lipid metabolism. Our understanding of the molecular biology, physiology and evolution of these alternative hGHs forms, will depend on the future supply of the

recombinant versions for comparative studies.

Here we report isolation, cloning and functional expression of both pituitary hGH cDNAs. We have integrated very simple cloning and production strategies, and evaluated the in vitro adipogenic activity of the cDNAs encoded hormones.

MATERIALS AND METHODS

Restriction and modification enzymes were purchased from Bethesda Research Laboratories Inc. (BRL, Gaithersburg, MD) and New England BioLabs Inc. (Beverly, MA), and used as suggested by the supplier. [α^{32}P]-dCTP, ^{35}S-dATP and L-[^{35}S]-methionine were purchased from Amersham Intl. (Buckinghamshire, England). The enzymatic kit for cDNA synthesis was obtained from BRL and the sequencing kit (sequenase) was purchased from United States Biochemical (Cleveland, OH)

Total cytoplasmic RNA was isolated from pituitary gland by the thiocyanate-phenol-chloroform technique [9]. Quantity and quality of RNA preparations were determined spectrophotometrically and corroborated by agarose gel electrophoresis [10]. Poly(A)$^+$ RNA was subsequently selected by oligo(dT)-cellulose chromatography [11]. The poly(A)$^+$ RNA was converted to cDNA, digested with AatII and XmaI and cloned into the similary cut pUC19 plasmid. DNA samples obtained during the procedure were

electrophoresed in a 1.4% agarose gel, transferred to nylon membrane (Gene Screen Plus) and analyzed by Southern blotting [12] with an homologous ^{32}P-labeled [13] hPL cDNA probe [14].

DNA restriction fragments were purified from agarose or polyacrylamide gel slices. This was performed by electroelution or Geneclean (BIO101, La Jolla, CA) extraction [15]. Ligations, bacterial transformations and plasmid DNA isolations were carried out using standard protocols [16]. Recombinant plasmids carrying hGH cDNAs were characterized by restriction analysis and nucleotide sequencing [16].

COS-7 cells (a gift from T. Kuo) were adapted to grow in Dulbecco's modified Eagle's medium (Sigma Chemical Co., St Louis, MO) containing 1% fetal calf serum, (HyClone Laboratories, Inc., Logan, UT). They were maintained at 37°C with 5% CO_2. Plasmid DNA (7.5 µg/ 25 cm^2 culture flask) was transfected by the calcium phosphate method [17].

Northern blot analysis of total RNA obtained from transfected COS-7 cells and in vivo labeling of newly synthesized and secreted proteins were performed as previously outlined [18]. Quantification of hGH was achieved using a commercially available hGH radioimmunoassay (RIA) kit (Diagnostic Products Co., Los Angeles, CA). The test was performed as described [19]

directly on the tissue culture medium.

Samples of culture medium from COS-7 cells transfected with the different plasmids, were added to a final concentration of 30% (v/v) to confluent 3T3-F442A cell cultures supplemented with non-adipogenic medium [20]. After 7 days, cell cultures were fixed and stained with oil Red O for adipose conversion quantification. Control cultures were also maintained with non-adipogenic medium with or without 25 ng/ml met hGH [illegible].

RESULTS AND DISCUSSION

Preferential cloning of pituitary hGH cDNAs.

The computer aided inspection of published hGH cDNA sequences [21] resulted in the identification of unique restriction enzyme sites flanking the hGH gene coding region. At the 5' end of both hGH cDNAs, we chose an AatII site centered at codon -20 of the signal peptide (26 aminoacid aminoterminal extension). At the opposite end, we found a convenient XmaI site located four nucleotides after the stop codon. The ligation of pituitary cDNA digested with AatII plus XmaI, to the similarly cut large DNA fragment of pUC19, resulted in the integration of hGH cDNAs into recombinant plasmids. The success of these reactions, and thus of our preferential cloning strategy, was monitored by Southern blotting [12] as shown in Fig.1. Transformation of

competent Escherichia coli strain RRI with this ligated material, resulted in 18 colonies. Dot blot screening of plasmid DNA isolated from the bacterial colonies, allowed us to identify putative hGH cDNA in three clones. Digestion with diagnostic restriction enzymes and partial DNA sequencing (data not shown), confirmed the presence of hGH-22K cDNA inserts in two of these clones (designated as pDRhGH22K) and of the hGH-20K cDNA in the third clone (designated as pDRhGH20K).

Subcloning and expression of the hGH cDNAs.

We then proceeded to subclone both hGH cDNAs from their AatII to XmaI sites, into the efficient eukaryotic expression vector pAVE2 [22] which already harbors 3' end untranslated, polyadenylation signal and 3' end processing sequences from the hGH gene. These cDNA copies were not full-length. They lacked the 5' and 3' untranslated regions, as well as the first six codons of the signal peptide. To complete both cDNAs and thus generate minigenes, we used DNA pieces derived from: i) the hPL-3 gene (from the cap site to the PstI site towards the 3' end of the first exon) and ii) the hPL-3 cDNA from this PstI site to an AatII site at the beginning of the second exon (codon -20 of signal peptide, which is 26 aminoacids long). In spite of the tailoring of these gene pieces, the mature hGH proteins derived from these hybrid minigenes are coded entirely by

the hGH cDNAs. The resulting hybrid plasmids, named pAVE2hGH22K and pAVE2hGH20K, are diagrammed in fig.2.

After transfection of COS-7 cells with the pAVE2 derivatives as well as with positive (pAVE1hGH) and negative (pAVE2) controls [22], total cellular RNAs were obtained and analyzed by Northern blot [18]. As shown in fig. 3, the transiently expressed hPL-hGH hybrid RNAs were specifically detected by hybridization with the ^{32}P-labeled hPL cDNA probe.

Cell culture production of hGH 22K and 20K.

To verify the correct expression of the transfected novel minigenes, the production of secreted hGH by the transfected COS-7 cells was determined by RIA. Values of secreted hGH-22K averaged 1259 ng per 60 mm culture dish of transfected COS-7 cells. The average value for secreted hGH-20K was 41 ng per the corresponding cultured dish.

As shown above in the Northern blot experiment, the hybridization signals given by the RNAs isolated from cells transfected with either pAVE2hGH22K or pAVE2hGH20K, are similar in intensity. This indicates that in our transfection assays, both minigenes are similarly efficient in generating mature mRNAs. In other studies it has been shown for the transiently expressed hGH gene, that the mRNA expression levels are directly reflected in hGH protein levels [23]. Thus, one explanation for having

such surprisingly low RIA values for hGH-20K, could be a poor recognition of this variant by the highly specific RIA. Similar results were found by Lewis et al. (1978) who provided evidence that hGH-20K was only one-third as active as the 22K hGH in their RIA [6].

To test this hypothesis, we decided to visualize both hGHs expressed and secreted into the medium used in the transfection assays. The proteins synthesized by the transfected cells were labeled with L-[^{35}S] methionine and those secreted into the culture medium were resolved by SDS-PAGE. The secreted proteins were then detected by autoradiography [18]. As it can be seen in fig. 4, the cells transfected with pAVE1hGH, which bears the hGH-N gene expressed hGH-22K abundantly; but, the 20K form derived from it was expressed at a lower proportion. The hGH-22K minigene behaved as the hGH-N gene directing the expression of large amounts of the 22K form of hGH, but in this case no hGH-20K was observed, as expected. On the other hand, the cells transfected with the hGH-20K minigene expressed the hGH-20K at levels above those found in cultures transfected with hGH-N gene, but below those of the hGH-22K produced by either of the other two recombinant plasmids. We still do not have an explanation for these differences in expression between the 22K and 20K hGH minigenes.

Adipogenic activity of the recombinant hGHs.

Further confirmation of the authenticity of secreted hGHs was obtained by evaluating their ability to specifically promote differentiation of 3T3-F442A preadipocytes cells to adipose cells [2]. Results obtained studying the biological activities of hGH variants, indicated that measurement of adipogenic activity is a much more sensitive assay for hGH activity than the measurement of body weight increase [24]. Control 3T3-F442A cell cultures supplemented with met(hGH)^{bac} (25 ng/ml) showed a 100% differentiation. Those cells supplemented with media samples from cultures transfected with pAVE2hGH22K also presented a significant proportion of differentiated cells (see table I). On the other hand, cultures exposed to medium containing hGH-20K secreted by transfected COS-7 cells, reached lower levels of adipose conversion (see table I). These results agree with the hGH-22K and hGH-20K protein levels detected by autoradiography (see above). In addition, they were similar to those obtained in experiments using pAVE1hGH as positive control (data not shown). The non-adipogenic medium from cells transfected only with the vector (pAVE2) did not stimulate this diferentiation process (table I).

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Figure 1. Southern blot analysis to confirm the preferential cloning of pituitary hGH cDNAs. DNA samples obtained during the cloning procedure of the hGH cDNAs were size fractioned, transferred to nylon membrane and hybridized with a radiolabeled hPL cDNA probe. Lane cDNAs: total pituitary cDNA; XmaI+AatII: total pituitary cDNA digested with AatII and XmaI; Cloning: ligation products of the AatII and XmaI-digested pituitary cDNA with similarly treated pUC19 vector; Vector: AatII and XmaI-digested pUC19 (negative control); Control: hPL cDNA (positive control) and M: 1kb Ladder. Left-hand= 1.4% agarose gel stained with ethidium bromide; right-hand= autoradiography.

Figure 2. Construction of pituitary growth hormone expression vectors. The plasmids pDRhGH20K in A and pDRhGH22K in B along with an intermediate plasmid (C) constructed in our laboratory containing a hPL-bGH-hGH hybrid minigene were used for the construction of pAVE2 derivatives vectors. A series of isolated DNA fragments (F,G,H and I) were assembled in different combinations in reactions catalyzed by T4 DNA ligase. The pAVE2hGH20K (D) was constructed with the fragment F from the coding sequence of 20K hGH cDNA (seventh codon of pre-hormone up till four nucleotides downstream termination codon) and the fragments H and I from pAVE2bGH, which lack the corresponding coding sequence of the hPL-bGH-hGH hybrid minigene. Like wise, pAVE2hGH22K (E) was constructed using fragment G instead of fragment F. Restriction sites with an asterisc are not unique in the maps shown. Stippled box, hGH cDNAs; open box, bGH cDNA; horizontally striped box, hPL-3 gene, vertically striped box, hPL-3 cDNA; inclined striped box, hGH gene 3' end. Sizes are not drawn at scale.

Figure 3. Northern analysis of hGH mRNA levels in COS-7 cells transfected with various hGH expression vectors. Ten μg of total RNA from transfected COS-7 cells were electrophoresed in a 1.2% denaturing phosphate-agarose gel and transferred to nitrocellulose membrane. The probe was the radiolabeled hPL cDNA. The individual expression vectors which were transfected are indicated at the top of the autoradiography. H y L represent lanes containing total DNA from human pituitary gland and rat liver, respectively. Lane labeled C represents total RNA of mock-transfected cells. For simplicity, we used pAVEhGH22K and pAVEhGH20K instead of pAVE2hGH22K and pAVE2hGH20K as the names of these recombinant plasmids.

Figure 4. Detection of the radiolabelled recombinant proteins encoded by hGH chimeric plasmids. Ten μg of DNA from the indicated expression vectors were transfected into COS-7 cells and the synthesized proteins were labelled with L-[^{35}S]methionine for 4 h. A 200 μg of protein sample was analyzed by electrophoresis on a 5-13% discontinuous polyacrylamide (SDS) gel. Gels were dried and subjected to autoradiography. The molecular weights of hGH recombinant proteins are indicated by arrows on the right.

Table I. Stimulation of adipose conversion by recombinant hGH's secreted by transfected COS-7 cells.

Plasmids	Adipose Conversion * (%)
None	0.0 ± 12.6
pAVE2	0.0 ± 1.7
pAVEhGH22K	100.0 ± 11.5
pAVEhGH20K	66.4 ± 4.5

* Values representing each data are average and standard deviations from three independent experiments.

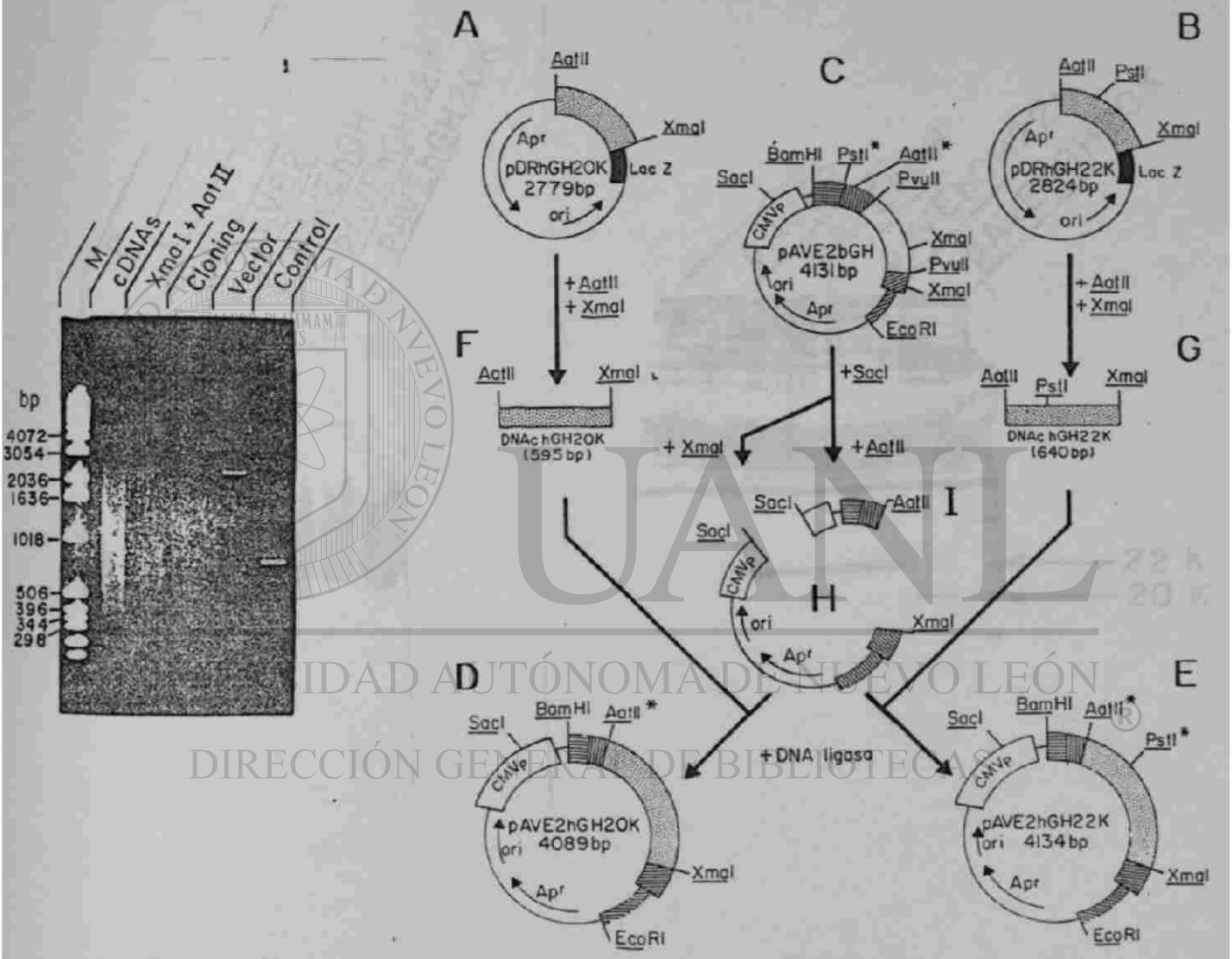

Figure 1

Figure 2

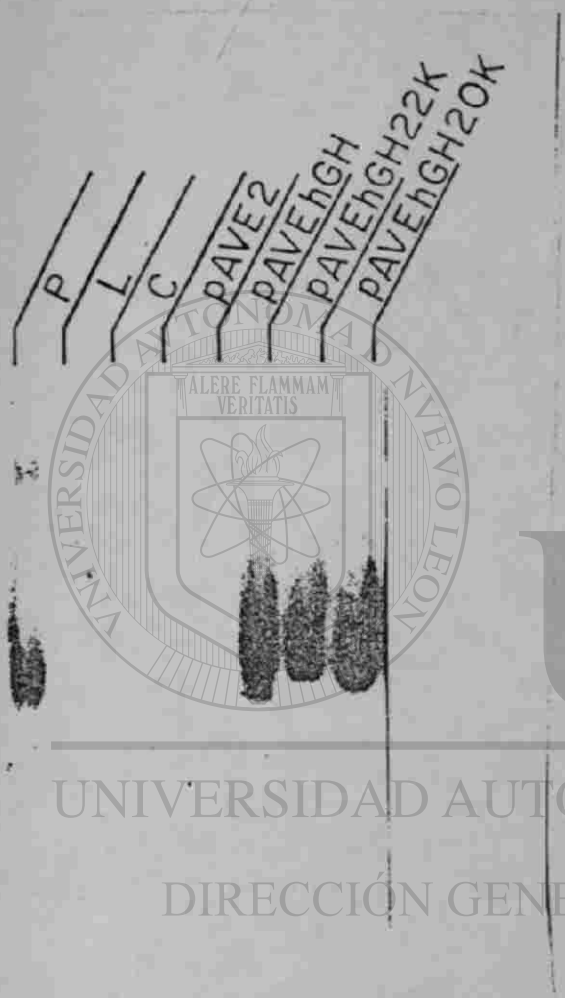

Figure 3

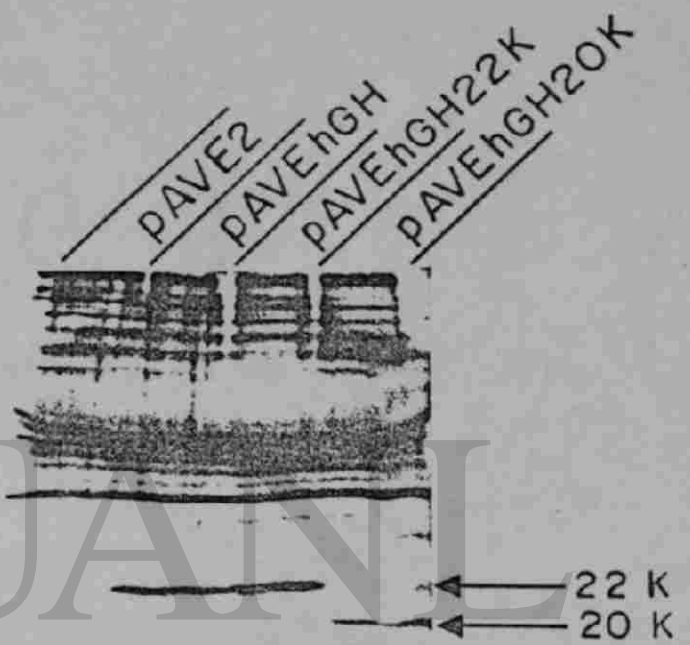

Figure 4

ANEXO B

Coding potential of transfected human placental lactogen genes

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ABSTRACT

We have joined the promoter-less sequences of the three hPL genes (hPL-1, hPL-3 and hPL-4) to strong transcriptional control elements. *in vivo* ^{35}S-labeled proteins from the culture medium of cells transfected with the genes were resolved on SDS-polyacrylamide gels. The presence of characteristic labeled bands, visualized by autoradiography, determined that hPL-4 and hPL-3, but not hPL-1, contribute to the production of mature hPL. In these experiments hPL-3 expressed more RNA and protein than hPL-4. By exchanging the first two exons among hPL and hGH genes, we determined that the abundance of chimeric proteins depended on the genetic origin of the first two exons. Finally, we found evidence indicating that the splice mutation (G—A) at the beginning of the second intron of hPL-1, is not the only cause of the apparent lack of inactivity of this gene, since its reversion does not restore expression.

INTRODUCTION

The human growth hormone-placental lactogen gene family is a multigene complex containing two human growth hormone (hGH) and three human placental lactogen (hPL, also known as chorionic somatomammotropin; hCS) genes (1,2). The entire gene cluster is located on the long arm of human chromosome 17 at bands q22 – 24 (3). The genes display the following 5' to 3' arrangement: hGH-N, hPL-1 (or hPL-L, L for like), hPL-4 (also known as hCS-A or hCS-1), hGH-V and hPL-3 (also named hCS-B or hCS-2). The best characterized protein products of this family are the secreted hGH and hPL polypeptides containing 191 amino acids. They are produced in the pituitary gland and placenta, respectively.

Recombinant DNA analysis has revealed a paradox in the coding potential of the hGH and hPL genes. The hGH-N gene, both *in vivo* and *in vitro*, generates through differential splicing of its primary transcription product, 22 kDa (90%) and 20 kDa (10%) forms of hGH (4,5). The hGH-V gene, whose expression has been demonstrated only in the placenta and in a single human pituitary tumor (6), has recently been confirmed to also generate *in vitro* a 22 kDa form. However, no 20 kDa protein derived from this gene has been detected (7). cDNA cloning and DNA sequencing also have revealed the existence in placenta of a second type of hGH-V mRNA. By retaining an in-frame fourth intron, this new mRNA is predicted to encode a mature protein of 26 kDa (8).

A completely different situation is observed with the hPL genes. The hPL-4 and hPL-3 genes have been found to be active in term placenta. In addition, their cDNAs have been cloned and sequenced (9). Their mRNAs are slightly divergent in nucleotide sequence. The encoded pre-hormones of these two mRNAs differ in a single amino acid position within the signal peptide (at position −24, hPL-3 codes for alanine while hPL-4 codes for proline). Yet, the mature hormones are identical. The third gene (hPL-1), is presumably nonfunctional, since it contains a mutation (G—A) at the 5' or donor splice site of the second intron. Transcripts derived from it have not been detected (9, 10). Therefore, while two hGH genes generates at least four different hormones, the sequences of the three hPL genes predict the synthesis of a single form of mature hPL hormone.

The sequence of both hPL-3 and hPL-4 genes and their cDNAs, predict that they might contribute to the placental production of hPL. However, since the mature proteins expected to be derived from them are identical, it is impossible to distinguish their gene(s) of origin. No evidence has been obtained demonstrating that the expression of either gene actually specifies the mature hPL protein.

In this study we performed an analysis of the *in vitro* expression products of all hPL genes. We specifically addressed the question of whether or not the hPL-3 and hPL-4 genes, known to be transcriptionally active in term placenta, produce mature hormones. These two genes, at different expression levels, were found capable of producing an intrinsic hPL protein. Experiments were also designed to determine if the splice point mutation at the beginning of the first intron of the hPL-1 gene, is the only cause of its apparent lack of expression.

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MATERIALS AND METHODS

Recombinant DNA constructions and preparation of plasmid DNA

Restriction and other enzymes were obtained from commercial suppliers and used according to their manufacturers instructions. The isolation of hGH and hPL genes have been previously reported (11). pNUT, constructed by R. Palmiter *et al.* (12) which already carries the hGH-N structural gene in front of the metallothionein promoter, was a generous gift. The hPL genes cloned in pSV2gpt (13) were kindly provided by G. Saunders. DNA restriction fragments were purified from preparative agarose or polyacrylamide gels. This was performed by electroelution, or by glass bead extraction (14) from the agarose gel slices. hPL and hGH promoter-less genes were subcloned into pNUT. The large *Bam*HI to *Eco*RI fragment of pNUT, was ligated to DNA fragments carrying the genes of interest. The genes consisted of sequences from their naturally occurring *Bam*HI site (except hPL-1; see Results) at nucleotide +2, to a natural or artificial (linkers) *Eco*RI site located several hundred nucleotides downstream of the polyadenylation signal. To construct our negative control, pNUT(−), we took advantage of the presence, in pNUT, of two *Xma*I sites. They flank hGH coding sequences: one artificial site is present at position −4, while the other is a natural site located four nucleotides downstream of the termination codon. By cutting with *Xma* I, diluting and ligating back, we obtained the derivative of pNUT lacking the hGH structural gene: pNUT(−).

Ligations, bacterial transformations and plasmid DNA isolation and characterization were carried out using standard protocols (15). Recombinant plasmids carrying all hGH, hPL or hybrid genes were characterized by digesting their DNAs with several diagnostic enzymes, by Southern blotting (16) or nucleotide sequencing (17).

Cell culture, DNA transfection, isolation of RNA and labeling of secreted proteins

COS-7 cells (a gift from T. Kuo) were adapted to grow in Dulbecco's modified Eagle's medium (Sigma chemical Co, St Louis MO.) containing 1% fetal calf serum (FCS), (Hyclone Laboratories, Inc. Logan, Utah). They were maintained at 37°C with 5% CO_2. By lowering the FCS concentration we could precipitate and analyze larger volumes of media. Plasmid DNA (7.5 μg/ 25 cm^2 culture flask) was transfected by the calcium phosphate method (18). We evaluated transfection efficiency performing CAT assays or through radioactivity counting of RNA hybridizated with the DHFR probe in slot blots. The CAT assays were carried out on a fraction of cultured cells or the entire culture co-transfected with both the test plasmid and pCMVCat (19).

Total RNA was recovered by the guanidinium thiocyanate-phenol-chloroform technique (20). Quantity and quality of RNA preparations were determined spectrophotometrically and corroborated by agarose gel electrophoresis (15).

To label newly synthesized and secreted proteins, 48 h after cells transfection the previously mentioned medium was replaced for a methionine-free medium containing 1% dialyzed FCS and ^{35}S-methionine (Amersham Intl, Buckinghamshire, England). *In vivo* labeling of newly synthesized proteins was performed by extending the incubation period for an additional 4 h. We labeled with 12.5 μCi of ^{35}S-methionine per ml of medium. The incubated medium was removed from culture flasks and stored. Since, the genes under study code for secreted proteins, we recovered their expressed products from 150 and 300 μl aliquots of the media by precipitating twice with four volumes of cold acetone. Subsequently, we dissolved the recovered proteins in layering buffer for SDS-polyacrylamide gel electrophoresis (21).

Southern blotting, Northern analysis, visualization of labeled proteins and radioimmunoanalysis

^{32}P-dCTP was purchased from Amersham Intl. (Buckinghamshire, England). Hybridization of DNA in nitrocellulose membrane was carried out as described by Southern (16). RNA was denatured and resolved according to size by agarose gel (22) electrophoresis. Once the above was performed, they were transferred to nitrocellulose sheets and hybridized to the probe (23). Both hybridization techniques used as probe, a 550 bp *Hae*III fragment of hPL cDNA (24) labeled with ^{32}P-dCTP by the technique of random primers (25).

Protein samples dissolved in layering buffer were boiled for 2 min and applied to 5−13% discontinuous polyacrylamide gels (21). Gels were placed on filter paper and dried under vacuum at 80°C. The dried gels were exposed to X-ray films at room temperature. Quantification of hGH was achieved using a commercially available hGH radioimmunoassay kit (Diagnostic products Co., Los Angeles, CA).

RESULTS

A new set of high expression plasmids for hPL structural genes

pNUT, contains the SV40 enhancer and metallothionein promoter directing the transcription of the promoter-less hGH-N gene (figure 1A). In addition, it efficiently expresses hGH in cell culture (12). We found, by radioimmunoassay, that COS-7 cells transfected with pNUT by the calcium phosphate method (18), yielded extracellular hGH values averaging 700 ng per 25 cm^2 culture flask.

We transferred the structural sequences (promoter-less) of all the hPL members of the hGH-hPL multigene family into pNUT (see figure 1B). This was accomplished by simply replacing the hGH-N gene structural sequences present in pNUT, for the corresponding sequences of the hPL genes. However, because hPL-1 gene lacks the convenient *Bam*HI site used for the transfer, we constructed a hybrid gene between hPL-1 and hPL-3 genes to provide it with such a site. The hybrid consists of the *Bam*H1−5′ end flanked first exon, first intron, and part of the second exon of the hPL-3 gene. The rest consists of hPL-1 sequences from the *Pvu*II site, within the second exon, to the *Eco*RI site at the 3′ end of the gene. Having constructed this hybrid allowed us to not only gain the useful *Bam*H1 site, but also allowed us to retain intact the second exon/second intron boundary of hPL-1. This area includes the donor splice mutation of interest, previously identified as potentially being the cause of lack of hPL-1 gene expression. From here on, this hybrid gene will be used instead of the hPL-1 wild-type gene. The recombinant plasmids were characterized by digestion with restriction enzymes (figure 1C) and by Southern blot analysis (figure 1D).

Expression of transfected hPL genes at the protein level

The figure 2 autoradiography reveals that cells transfected with the plasmid carrying the hGH-N structural gene (pNUT), secreted characteristic 22 kDa and 20 kDa forms of hGH (lane: hGH-N). The lane containing media from pNUThPL-1 transfected cells (lane: hPL-1), does not exhibit bands of at least the same intensity

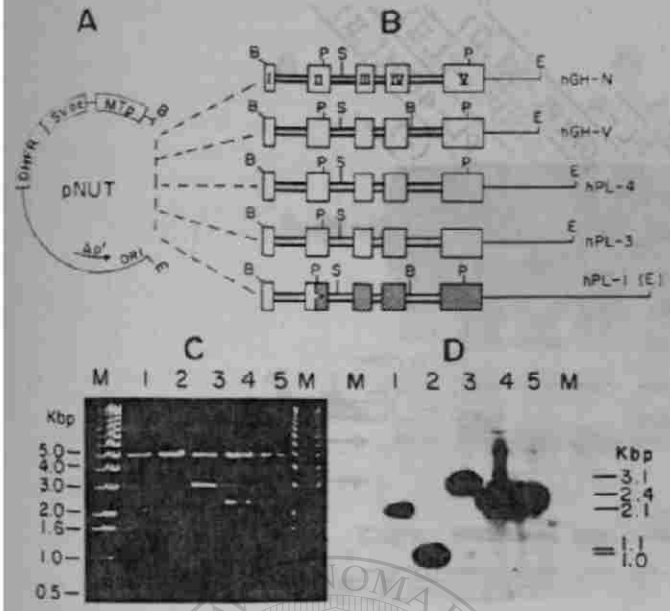

Figure 1. Construction of the hPL and hGH expression plasmids. To subclone hPL genes into pNUT(A), we replaced the hGH-N gene sequences in pNUT for the corresponding ones of hPL genes (B). The drawings at the top illustrate the maps of the expression plasmids (A and B). Both, restriction enzyme (C) and DNA hybridization (D) analysis, confirmed the identity of the new expression plasmids. Lanes in C and D show gel and Southern autoradiography of plasmids cut with *Eco*RI plus *Bam*HI and are as follows: 1, pNUT; 2, pNUThGH-V; 3, pNUThPL-1; 4, pNUThPL-3; and 5, pNUThPL-4. M corresponds to molecular weight (in kbp, at the left of C) DNA standards. Only the sizes of hybridizing bands are indicated (in kbp at the right of D). The hPL-1 is really a hybrid of hPL-3 and hPL-1 (see Materials and Methods section for explanation). The pNUThGH-V construct, presented here, was not analyzed. MTp = mouse metallothionein promoter; SVpe = SV40 early promoter; DHFR = structural gene for dihydrofolate reductase; Ap^r = B-lactamase gene; ORI = pBR322 origin of replication; B = *Bam*HI; P = *Pvu*II, S = *Sac*I, E = *Eco*RI. Boxes represent exons.

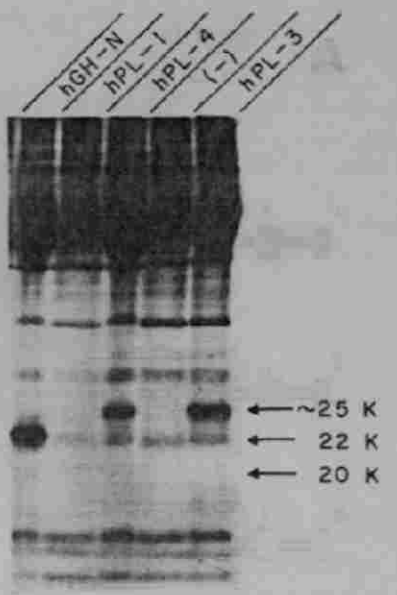

Figure 2. *In vitro* production of secreted proteins by hGH-N and hPL genes. Media from COS-7 cells transfected with each of the plasmids and incubated in the presence of ^{35}S-methionine, was analyzed by discontinuous SDS-polyacrylamide gel (5%−13%) electrophoresis and autoradiography. The gene present in each plasmid used for transfection is indicated at the top. The (−) symbol identifies the media from cells transfected with the vector alone [pNUT(−)]. Sizes of characteristic hPL and hGH bands are indicated in kDa (K) at the left.

and size of hGH. Media from cells transfected with pNUThPL-4 (lane: hPL-4) presents a less prominent band, but of slightly greater size (close to 25 kDa) than that of hGH. Finally, the only hPL-3 form observed (lane: hPL-3), is of hPL-4 size. However, its intensity is that of the 22 kDa form of hGH.

Differences in the expression of hPL proteins

As noticed above (figure 2), while the hGH-N gene gives rise to a prominent band of approximately 22 kDa, hPL-3 and hPL-4 genes express proteins of slightly greater size (~25 kDa). Furthermore, and consistently throughout several independent experiments, the hPL-4 band always appeared weaker than the hPL-3 band. We were interested in investigating the cause of such heterogeneity in the expression levels of these genes.

To approach this problem, we chose to study the cell culture production of extracellular hPL-hGH chimeric proteins resulting from the transient expression of a new hybrid gene pair. These hybrids possess the first two exons from hPL-3 or hPL-4 genes, and sequences of hGH-N gene that conform the remaining part of their structure. We named these hybrids GH(PL-3:I,II) and GH(PL-4:I,II) respectively (see map in figure 3A). By comparing both hybrid genes, differences were observed in the expression levels of GH(PL-3:I,II) versus GH(PL-4:I,II) chimeric proteins [figure 3B; compare lanes labelled GH(PL-3:I,II) and GH(PL-4:I,II)]. Same results, we might add, as with the proteins derived from normal non-hybrid hPL-3 and hPL-4 genes [see lanes labeled hPL-3 and hPL-4 in figure 2 and labeled PL-3 and PL-4 in figure 3B).

Consistently, we obtained less protein when sequences from the first two exons of hPL-4 gene were present. On the contrary, we detected more hGH-like protein when the first two exons were from hPL-3. Moreover, when the last three exons were from hPL-4 gene, and the first two exons were from hGH-N (see map of this chimeric at bottom of figure 3A), the band intensity resembled that of hGH-N protein [figure 3B, lane PL-4(GH:I,II)]. Therefore, relative abundance of hPL protein products is a function of the first two exons.

To further investigate the different levels of *in vitro* expression observed for these active hPL genes, we carried out estimations of the relative abundance of their RNA transcripts. Using slot blot analysis, we found approximately ~8-fold more RNA hybridizable to our probe from the total RNA isolated of cells transfected with the hPL-3 structural gene sequences, as compared to hPL-4 (figure 4). This same result was observed even when only the two first exons of hPL-3 were contributing to a hybrid gene (D.R.-P. and H.A.B.-S., submitted. Therefore, the higher observed hPL-3 protein expression, seems to be a consequence of having more RNA derived from the hPL-3 gene.

Dissecting the putative hPL-1 pseudogene

Next we, decided to test if the donor splice site point mutation at the second intron of the hPL-1 gene, was the only cause of the apparent inactivity of this gene. Comparing nucleotide sequences at the second exon/second intron border area, among the active hPL-3 gene and the putative hPL-1 pseudogene, revealed that we could easily exchange this region between these two genes. We found *Pvu*II and *Sac*I sites located 30 bp upstream and 86 bp downstream respectively, from the mutation site (figure

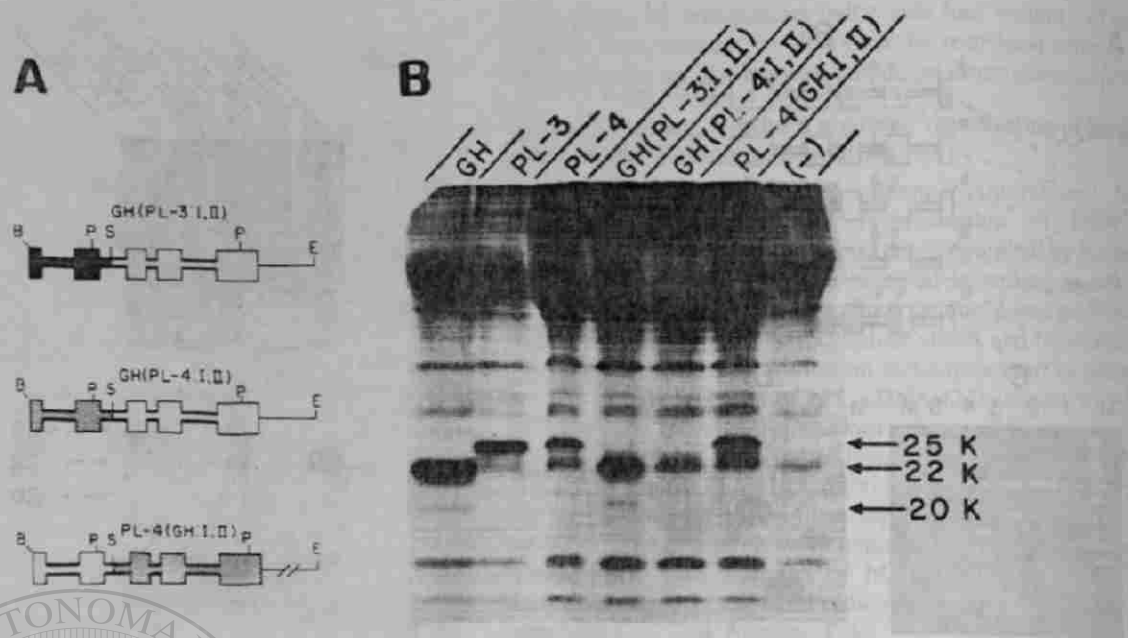

Figure 3. Diversity in size and abundance of protein expression products from, hPL and recombined hPL-hGH genes. Maps in A demonstrates the structure of the hybrid genes; their are composed of hPL-3 (solid) or hPL-4 (hatched) and hGH (open) gene portions. As in the previous figures, dealing with protein analysis, each lane in B corresponds to media of cells transfected with plasmids carrying the genes indicated at the top. The negative control, (−) symbol, distinguishes the media from cells transfected with pNUT(−) (vector alone). The new bands are pointed by arrows. Size is also in kDa (k).

1B). In addition to the splice site point mutation, this region flanked by *Pvu*II and *Sac*I of approximately 120 bp, differs within these two genes in only three nucleotide positions. These differences, are located inside the intron at nucleotide positions of little importance for the pre-mRNA processing (26). The *Pvu*II-*Sac*I region encompassing the second exon/second intron boundary, was exchanged between the putative hPL-1 pseudogene and the active hPL-3 gene. This manipulation gave rise to two new hybrid genes. We named these new recombinants as hPL-1r and hPL-3m, for 'repaired' hPL-1 and 'mutated' hPL-3 genes, respectively.

To determine the effect on the splice mutation expression, we transferred these hybrid genes, once constructed, into pNUT. Both the hybrid and non-hybrid genes (controls) were introduced into COS-7 cultured cells. Figure 5B demonstrates the results obtained from the analysis of *in vivo* labeled secreted proteins. In lane labeled PL-3 which corresponds to media of cells transfected with pNUThPL-3, we easily detected an hPL band. On the other hand, cells transfected with either pNUThPL-1 (lane: PL-1) or pNUThPL-3m (lane: PL-3m), revealed the absence of obvious hPL bands. Likewise, we could not observe an hPL band from the media of cells transfected with the new repaired hPL-1 gene (figure 5B, lane: PL-1r).

A dramatic effect of the splice donor site mutation at the second intron of hPL-1 was also observed; when, we constructed and used in comparative studies, a new hybrid gene. It consists of hPL-3m sequences, joined at the unique *Sac*I site (within the second intron) to the remaining portion of hGH-N gene (bottom of figure 5A). This new hybrid was studied in conjunction (as positive control) with the previously mentioned hybrid gene created between hPL-3 and hGH-N.

A prominent hGH-like (similar in hGH size) protein was found in the media of cells transfected with the non-mutant hybrid gene [see map of GH(PL-3:I,II) in figure 5A, and expression results in lane: GH(PL-3:I,II) of figure 5B]. This positive control gene

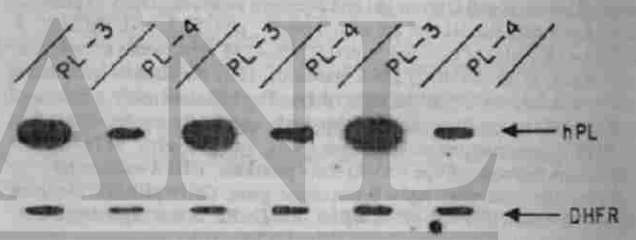

Figure 4. Slot Blot analysis of hPL-3 and hPL-4 RNA expressions. Total cellular RNAs were obtained from three independent experiments in which COS-7 cells were transfected with either pNUThPL-3, or pNUThPL-4. 3μg of RNAs were applied to the slots and hybridized with a DHFR cDNA probe. To determine efficiencies of transfection, the slots were cut and its radioactivity contents measured by liquid scintillation counting. RNAs in amounts compensating transfection efficiencies, were then hybridized separately with the DHFR and with an hPL cDNA probe (arrows). Extent of hybridization was also assessed by counting radioactivity in each slot.

also carries the alternative splice acceptor site present inside exon 3 of the hGH-N gene (4). Splicing exons 1 and 2 to this alternative acceptor site, results in the 15 amino acid internal deletion characteristic of the 20 kDa form of hGH. The presence of a new minor band of 20 kDa together with the 22 kDa band, in the media of cells transfected with pNUTGH(PL-3:I, II), demonstrates that the alternative splicing mechanism also occurs in this hybrid gene.

No hPL or hGH-like proteins were observed (last lane in figure 5B) when the second member of this pair carrying the hPL-1 mutation was used in the transfection.

Effect of the splice mutation of hPL-1 gene in mRNA production

We wanted to know whether the apparent absence of hPL protein from cells transfected with the pNUThPL-1 plasmid, was a

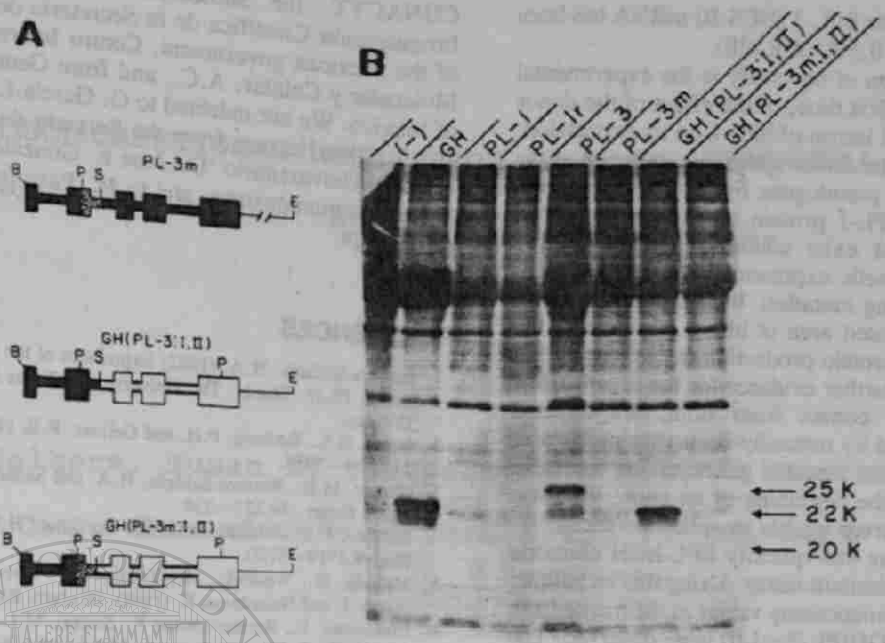

Figure 5. Effect of the donor splice site mutation of hPL-1 gene on the production of secreted proteins. Cells transfected with the different plasmid carrying the hybrid genes indicated at the top, were analyzed for the presence of secreted labeled proteins in their media. Hybrid genes were constructed as described in the Material and Methods and Results sections, and for purpose of clarification, maps of key hybrid genes are shown in A. Characteristic non-chimeric and chimeric proteins were visualized as described in Material and Methods section, gene portions in black, hPL-3 gene; stippled, hPL-1; and open, hGH. Only important recognition sites for restriction enzymes are indicated. B=*Bam*H1, P=*Pvu*II, S=*Sac*I and E. *Eco*RI. Arrows in B indicate the size in kDa (k) of the new proteins originated from plasmids transfected. (−)=media from the negative control cells.

consequence of having no RNA derived from the PL-1 gene. We performed a Northern blot analysis (23) of the total RNA extracted from transfected cells. We included as a positive control, RNA isolated from human placenta and pituitary gland. In addition, as another positive control, we included RNA from cells tranfected with the plasmid carrying the hPL-3 structural sequences. The results of this analysis are presented in figure 6. Cells transfected with the plasmid carrying the hPL-1 gene lacked hPL specific RNAs (figure 6, lane: PL-1). We observed a dramatic reduction in the hybridizable RNA content from cells transfected with the plasmid carrying the hPL-3 mutated gene (figure 6, lane: PL-3m); as compared with, the wild type hPL-3 gene acting as control (lane: PL-3). Finally, when using pNUThPL-1r, we unexpectantly observed only a faint reappearance of hPL mRNA (figure 6, lane: PL-1r).

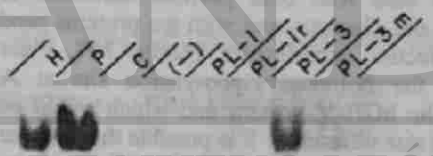

Figure 6. RNA expression effect of the donor splice site mutation of hPL-1 gene. The figure demonstrates the results of the Northern blot analysis (23) practiced to total RNA (10 μg), isolated from cultured cells transfected with the indicated (at top) plasmids. H and P represent lanes containing total RNA from human pituitary gland (2 μg) and placenta (3.8 μg), respectively. The (−) symbol correspond to RNA (10 μg) isolated from cells transfected with the pNUT (vector alone). Lane labeled C represents total RNA of mock-transfected cells.

DISCUSSION

DNA cloning and sequence studies have lead to the isolation of cDNA clones for all hGH and hPL genes except hPL-1. However, experiments directed to demonstrate that each of the mRNAs corresponding to the identified cDNAs indeed end up as proteins, have been few for hGH (7, 27) and none for hPL genes. The reintroduction of cloned genes into cultured cells, by DNA transfection (18), is a valuable method to identify and dissect sequences required for gene function and their mutations. We chose this approach to determine the coding potential of all hPL genes. To achieve our objective, we forced the *in vitro* expression of all the hPL promoter-less genes, by joining them to the strong heterologous transcriptional control sequences present in pNUT (12).

The new results of the present study demonstrate for the first time that the hPL-4 and hPL-3 genes, but not hPL-1, contribute to the production of mature hPL. Here we also demonstrate that in spite of being highly similar, the structural sequences of these genes respond differently to the same heterologous promoter. Each of these two genes give rise to one protein. Although the secreted proteins expressed by these genes have identical amino acid sequence, they differ at their expression level. As a consequence of this finding, we designed exon exchange experiments to gain new insights in the understanding of this phenomenon. The same result was seen when only the two first exons of the genes were contributing to a hybrid gene. The observed higher hPL-3 protein expression seems in part to be a consequence of having more RNA expression from the hPL-3 gene sequences. This *in vitro* findings do not resemble what occurs *in vivo*, while the hPL-4 mRNA (HCS-A) accounts for

3% of the placenta mRNA, the hPL-3 (HCS-B) mRNA has been estimated to represent only 0.5% of it (28).

Finally, a third contribution of our study is the experimental demonstration, also for the first time, of the effect of the donor splice mutation at the second intron of hPL-1 on its expression. In spite of having replaced the donor splice site mutation at the second intron of the hPL-1 pseudogene by normal sequences, we could not observe an hPL-1 protein secreted into the cell medium. Thus, there must exist additional mutations that contribute to this lack of genetic expression. There is no doubt of the severity of this splicing mutation. Indeed we prove here that by introducing the mutated area of hPL-1, into either the hPL-3 or the hGH-N gene, protein production from these hybrid genes is severely reduced. Further evidence for the severe effect of this type of mutation comes from both, site-directed mutagenesis studies (26) and by naturally occurring mutants of β-globin genes. In both cases, mutated genes having the same change of a G for an A at the beginning of an exon, give rise to a messenger RNA precursor unable to splice correctly.

We recently found that we can quantify hPL-hGH chimeric proteins using an hGH radioimmunoassay. Using this technique, non-detectable hGH radioimmunoassay values in the media from cells transfected with pNUTGH(PL-3m:I,II) were observed. The media of cells corresponding to the control experiment, where GH(PL-3:1,II) gene was used, gave hGH RIA values of about half of those of pNUT. Five-fold lower RIA values were found for the expression of GH(PL-4:I,II) as compared to GH(PL-3:I,II) (D.R-P., and H.A.B-S, submitted).

We have no explanation for the difference in size observed in the electrophoretic analysis of hGH and hPL proteins. The difference was seen with both purified hormones from the pituitary gland and placenta, and with the proteins produced in the gene transfection experiments. Neither hPL-3 nor hPL-4 proteins have the N-linked glycosylation site at Asn-140, predicted for the hGH-V protein and which might otherwise account for this size difference. It is possible that the size change observed, may simply be accounted for by differences in protein-SDS interactions.

In conclusion, our analysis of the *in vitro* expression products of hPL genes demonstrates that hPL-3 and hPL-4 have the potential for contributing to mature hPL. It also provides evidence indicating that the hPL-1 gene has accumulated severe mutation(s), other than the donor splice site deffect at its second intron. Furthermore, through our study, we have identified the gene region between the capping site and the second intron as the origin of differences in expression levels seen here for hPL-3 and hPL-4. The results obtained with the hPL-hGH genes hybrids corroborate and strengthen our findings.

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ABBREVIATIONS

bGH, bovine growth hormone; bp, base pairs; CAT, chloramphenicol acetyl transferase; FCS, fetal calf serum; hCS, human chorionic somatomammotropin; hGH, human growth hormone; hPL, human placental lactogen; hPrl, human prolactin; kbp, 1000 base pairs; kDa, 1000 daltons; Pre-hPL, hPL precursor; SV40, simian virus 40; SDS, sodium dodecyl sulphate.

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TRANSCRIPTIONAL REGULATION OF HUMAN PLACENTAL LACTOGEN GENES

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The placenta is an important source of a diverse group of hormones, cytokines and growth factors necessary for continuation of pregnancy. Due to its continued growth and differentiation during gestation, the placenta represents a unique organ for the study of hormonal genes during development. The production of one hormone in particular, placenta lactogen (PL) also called chorionic somatomammotropin (CS), reflects the development of the placenta as blood PL levels rise throughout pregnancy peaking at term.

Human placental lactogen (hPL) is a member of a closely related gene family that includes growth hormone (hGH) and prolactin (hPRL). Though these genes are highly similar and have evolved from a common precursor they have dramatically different physiological functions and regulatory mechanisms. Recently, advances have been made in understanding the regulation of hPL production and its physiological significance. In this review we discuss the various physiologic actions of hPL during pregnancy, the evolution and structure of hPL genes and, the tissue specific regulation of hPL gene expression.

Structure and Production of hPL Protein

Human placental lactogen (hPL), also called chorionic somatomammotropin, is a single-chain polypeptide hormone of 22,000 Da. It is composed of 191 amino acids with two intramolecular disulfide bridges (1) but contains no carbohydrate residues (Fig. 1). The mature hormone is derived from a precursor of

25,000 Da, that has a 26 amino acid signal sequence cleaved from its amino terminal end (2). Josimovich and MacLaren (3) first defined human placental lactogen as a polypeptide hormone present in extracts of human term placentae and retroplacental blood, that exhibited both potent lactogenic activity in rodents and immunochemical cross-reactivity with human growth hormone.

The mature placenta is shaped like an oval disc of about 500-600 g. It develops from the trophectoderm of the implanting blastocyst as it invades the endometrium. Differentiation of the placenta leads to the formation of villi structures composed of an outer layer of multinucleate syncytiotrophoblast cells formed by the fusion of underlying mononuclear cytotrophoblasts. These cells encapsulate a central core of mesenchymal cells, macrophages, and capillary epithelium.

hPL can be detected by immunofluorescence in the syncytiotrophoblast five to ten days after implantation of the fertilized ovum (4,5) or 12-17 days postfertilization. Hoshina *et al.* (6) used *in situ* hybridization to localize hPL mRNA exclusively to the syncytial layer suggesting synthesis of hPL production does not begin until formation of fully differentiated syncytiotrophoblast. Furthermore, *in situ* hybridization studies showed that the concentration of hPL mRNA in individual syncytiotrophoblast cells remains constant throughout pregnancy (6) suggesting the increase in the total amount of hPL mRNA in the placenta between first and third-trimester results from an increase in the number of

syncytiotrophoblasts in the placenta. This is in agreement with data showing the proportion of syncytial cells in the trophoblast (6-8), and the mass of the trophoblasts increase during pregnancy (8). Therefore, the increase in hPL synthesis by the placenta during pregnancy is at least partly due to an increase in the number of hPL-producing syncytiotrophoblasts.

By the third week post-conception, hPL can be detected by radioimmunassay in the maternal circulation. Peripheral serum levels of hPL then rise throughout pregnancy (9). During the third trimester hPL production can reach 1-3 grams per day (10), accounting for 10% of placental protein production and 5% of total RNA in the placenta (11, 12). At these levels hPL is the most abundant peptide hormoe produced in primates. The concentration of hPL in the maternal blood at term ranges between 5 and 15 μg/ml (8, 13, 14). hPL is present in the fetal blood but its concentration at term is 300-1000-fold lower than that of the maternal blood (13, 15, 16-18). The half-life of hPL in the maternal serum is 10-30 minutes (10, 13, 15, 19, 20) which accounts for its rapid disappearance from maternal serum after delivery.

Physiological Significance of hPL

Although much is known of the structure of hPL, the levels of hormone present throughout pregnancy, and the tissues where hPL is found, the role of placental lactogen in regard to fetal growth and metabolism was until lately poorly understood (Fig. 2). Because hPL is structurally very similar to human growth hormone (hGH),

several studies were undertaken to determine if hPL acted as a growth hormone for the fetus. Earlier bioassays using postnatal tissues and lower primate species showed hPL to have 1% of the growth promoting activity of hGH (21). More recent studies however, demonstrate that hPL has a direct somatotropic and metabolic effect in isolated human fetal tissues. Although the release of hPL is heavily biased towards the maternal circulation, the concentration of hPL in the plasma from human fetuses at 12-20 weeks gestation ranges from 4-500 ng/ml (22) and values of 20-200 ng/ml have been found in the cord blood of the newborn infant (23), suggesting a possible anabolic role in the fetus. Hill and colleagues in a series of publications were the first to make an in depth investigation into the actions of hPL upon fetal growth. They found that hPL, but not hGH, stimulates amino acid transport, [^{3}H]-thymidine incorporation, mitogenesis, as well as somatomedin-C (SM-C) and insulin like growth factor I (IGF-I) release in cultured fibroblasts and myoblasts from human fetuses at 13-19 weeks gestation (24, 25). In *in vitro* studies, hPL was found to bind to fetal tissues at hPL concentrations comparable to those found in fetal plasma, and direct anabolic effects of hPL have been found at concentrations similar to those required for binding to fetal membranes (25). Additional evidence suggests distinct hPL receptors are found in human fetal skeletal muscle, whereas there was a deficiency of fetal muscle hGH receptors (22). Evidence was also obtained suggesting unique hPL and hGH receptors are present

in fetal liver. hPL and hGH both stimulated growth of fetal hepatocytes and SM-C/IGF-I release from fetal liver. It is thought that hPL stimulates DNA synthesis in fetal connective tissue and liver indirectly through the paracrine release of the growth potentiators SM-C/IGF-I (22, 24, 25). The relationship, if any, among the hPL concentration in the fetus, the number of fetal hPL receptors and the rate of fetal growth has not been established. However, there is evidence to suggest that the weight of the fetus in early and middle gestation correlates positively with the hPL-binding capacity of the fetal liver and to a lesser extent with the fetal plasma hPL concentration (22).

Aside from its possible function as a direct acting, growth promoting hormone, substantial evidence suggests hPL influences fetal growth in an indirect manner by altering maternal metabolism. The major role of hPL in human pregnancy is believed to be mediated by its action as an insulin antagonist (4, 26). Carbohydrate tolerance was reduced in diabetics by hPL treatment (27, 28) It is believed that this action of hPL may be responsible for the development of diabetic ketoacidosis in pregnant women. hPL has also been shown to increase the insulin response to a glucose load, (19, 20, 27), perhaps as a result of an increase in insulin resistance (29).

The effect of hPL which has been most studied is its role in regulating lipolysis. Some evidence suggests that hPL is indirectly lipolytic (20, 30). Adipose tissue explants and adipocytes, stimulated by hPL, release glycerol and/or

nonesterified fatty acids (31). hPL also stimulates glucose uptake, glucose oxidation to CO_2 and glucose incorporation into glycogen, glycerol, and fatty acids in adipocytes (32, 33). This suggests a model for hPL action on adipose tissue that includes hPL-mediated increases in the basal rate of lipolysis as well as increases in glucose uptake and utilization. These actions could be physiologically significant since in the fasted state the increase in the rate of lipolysis provides free fatty acids that can be utilized as a source of energy by the mother. This spares the required glucose for use by the fetus. In the fed state, when maternal blood glucose concentration is high, the increase in glucose uptake and utilization by adipose tissue ensures that energy stores, in the form of triglyceride, will be available during subsequent periods of fasting (34). There is support for this theory as a study of women fasting for 84-90 hours during the second trimester of pregnancy reported a 30-40% increase in hPL production (35). In a similar study, fasting in pregnant women caused a much greater mobilization of free fatty acids, in the form of ketoacids, than in non-pregnant controls (36). The ability to produce PL may, therefore, be a selective advantage to foraging animals where there is the possibility of starvation, because PL causes more efficient use of energy stores and higher probability of offspring survival.

Placental lactogen received its name due to its lactogenic activity *in vivo* when administered to rabbits and pigeons (3). There

is, however, no evidence for lactogenic activity of hPL in humans *in vivo*, although there is evidence of the presence of mammotropic activity during pregnancy. hPL has been reported to stimulate DNA synthesis in epithelial cells of benign human breast tumors *in vitro* (37) and in human breast tumors maintained in athymic mice (38). It has also been reported to stimulate growth of ductal epithelium in human mammary explants obtained during lactation (39). Thus, the role of hPL in human mammary gland may be to stimulate cell proliferation, rather than milk secretion.

Evolution of The hPL/hGH Gene Cluster

On the basis of amino acid sequence homology among hGH, hPL, and human prolactin (hPRL), it was proposed that the hormones evolved from a common precursor (40). Whereas hGH and hPL share identical amino acids at 167 of 191 positions for an 87% amino acid homology, hGH and hPL are only about 35% homologous to hPRL at the amino acid level (41, 42). At the nucleotide level hGH and hPL are again very similar as mRNAs from these genes are 93.5% homologous. The hPRL mRNA has 42 and 41% identity with those of hGH and hPL, respectively (43). It is thought that GH/PL and PRL diverged approximately 350 million years ago (44, 45) when a primordial gene was duplicated giving rise to two separate precursors, one for the hGH and hPL genes and the other for the hPRL gene. The hGH/hPL and hPRL precursor genes then segregated onto two different chromosomes, since the hPRL gene is located on chromosome 6 (46) and the

hPL and hGH genes are found on chromosome 17 (47). Further evidence for the evolutionary relatedness of the hPL and hGH is that all the hPL and hGH genes are structually similar having 5 exons and 4 introns (12, 48, 49) and comparison of nucleotide sequences between any of the genes shows they share 91-99% sequence identity throughout, including 500 bp upstream of the genes (43, 44, 49, 50). It is believed that PL arose after the divergence of rodents and primates 80 million years ago (51). Evidence for this theory comes from DNA sequence data suggesting that rat PL (rPL) is derived from rPRL, as rPL is more homologous with rPRL and hPRL (52% and 51%) than rGH, hGH, or hPL (34% for all genes) (52). Therefore, duplication of the hGH/hPL gene to give rise to the hPL gene must have occurred after the separation of the main orders of mammals, during the evolution of primates (53). It is thought that the genes of the hPL/hGH gene cluster arose from their common precursor within the last 10-15 million years due to recombination events involving moderately repeated sequences (44, 54). Of the 66 kb in the hPL/hGH gene cluster, 21% of the sequence consists of 48 *Alu* repeat sequences (54). The abundance of highly homologous DNA sequences may predispose this locus to chromosomal misalignment, generating unequal recombination events (54). It was proposed that the hPL/hGH locus evolved in three steps (Fig. 3) involving large sequence duplications which preferentially begin and end with *Alu* elements (43, 54). The first event is thought to have been a duplication of a single GH/PL gene to a two-gene locus

composed of ancestral GH and PL genes. An additional duplication of this two-gene locus generated a four-gene arrangement of the ancestral hGH-N, hPL_4/hPL_1, hGH-V and hPL_3 genes. In the final step, the ancestral hPL_4/hPL_1 gene duplicated about 5 million years ago to generate the modern hPL_4 and hPL_1 genes.

Because the hPL_4 and hPL_1 genes were the last to be generated they should be the most homologous genes of the cluster. This is not the case as hPL_4 and hPL_3 are more closely related in terms of seguence homology (2.7% divergence) than the hPL_1 and hPL_4 genes (6.7% divergence) (43). However, downstream from the fourth intron the hPL_1 gene becomes more similar to the hPL_4 gene. This supports the hypothesis that hPL_4 arose from duplication of the hPL_1 gene but that a recent gene conversion event with a breakpoint in the fourth intron caused the hPL_4 gene to be more similar to the hPL_3 gene.

hPL_3 (also referred to as hCS-B or hCS-2) and hPL_4 (also refered to as hCS-A or hCS-1) share 98% sequence identity and encode identical mature proteins, but have one amino acid difference in the signal peptide. hPL_3 contains an alanine at the third position from the amino terminus of the precursor peptide, whereas hPL_4 has a proline at this position (2). The other hPL gene, hPL_1 (also referred to as hCS-L or hCS-5), was found to be a pseudogene, as no mature mRNA is produced from this gene due to a G to A transversion in the 5' splice site of the second intron (43, 54). In contrast, both hGH genes produce viable proteins. Alternative splicing of the primary hGH-N (also denoted hGH_1) gene transcript (48) generates two mRNA species that encode the major

active 22 kDa hGH protein as well as a 20 kDa variant which has 15 amino acids deleted from hGH (55, 56). The hGH-V (also called hGH_2) gene encodes a processed polypeptide differing at 13 amino acids from hGH-N. As the amino acid differences are mostly non-conservative they are thought to result in changes in the properties of the protein (11).

Gene Deletions in the Locus

The hGH/hPL gene cluster was examined in two cases in which hPL was reduced or absent in maternal serum during pregnancy. In the case of a partial deficiency of placental lactogen (57), hPL_4 was deleted in the maternal allele while the paternal allele lacked the hPL_4, hGH_2, and hPL_5 genes. The level of hPL in the maternal serum was one-fourth of the normal value, suggesting a direct relationship between gene dosage and hPL concentration in the maternal serum. Expression from the residual hPL_5 gene did not appear to compensate for loss of the other hPL genes. A direct relationship between lower hPL protein levels and decreased hPL mRNA levels was demonstrated in another case of partial hPL deficiency (58). Complete deficiency of hPL is a rare condition. Only three cases have been reported where using radioimmunoassay, no hPL could be detected during pregnancy (59-61). In the case that has been investigated in detail, a homozygous deletion of the hPL_4, hGH_2, and hPL_5 genes was found in the affected child while other family members were heterozygous for the deletion (62). The hPL_1 gene was present in the affected child but its expression was

not assayed. A novel hGH-hPL hybrid protein was detected that might have been able to compensate for the absence of hPL (63). It is not known whether this peptide is biologically active or if it is present in other cases where there is no deletion of hPL genes. However, it is conceivable that the chimeric hPL/hGH peptide could assume hPL-like roles in the mother and fetus, sustaining normal growth and development during pregnancies complicated by a deficiency of hPL production (25) Therefore at this time, the absolute necessity of a protein having hPL activity during pregnancy is still unknown.

Tissue Specific Expression of hPL and hGH Genes

Though the two genes evolved from a common ancestor and are closely linked, the hGH and hPL genes are expressed predominantly in two separate tissues. Growth hormone is secreted by somatotrophs of the anterior pituitary, whereas placental lactogen is produced by syncytiotrophoblasts in the placenta (2,6,64). In an effort to determine the levels of mRNA production of the individual genes in the cluster, Chen *et al.* (54) estimated the extent to which each gene is transcribed by the percentage of recombinant bacteriophage found in pituitary and placental cDNA libraries that hybridize to probes corresponding to each gene. In this study it was found that hGH-N accounts for 3% of the mRNA produced in the pituitary, whereas hGH-V is not expressed in the pituitary, but comprises <0.001% of mRNA in the placenta. This

hGH-V placental expression may be explained by novel recombination events during evolution of the gene cluster that placed putative hPL transcriptional control regions 5' to the hGH-V gene (54). hPL_4 was found to be the most abundantly expressed hPL gene by a 6:1 ratio accounting for 3% of placental mRNA with hPL_3 comprising an additional 0.5% of the mRNA. Studies by Barrera-Saldaña *et al.* (2) determined placental levels of hPL_3 and hPL_4 more directly. Though highly homologous, hPL_4 and hPL_3 could be differentiated by an additional *Pvu*II restriction site present in hPL_4 and a 4 bp insertion within the hPL_3 gene. These differences were exploited in restriction enzyme digests of placental cDNA clones as well as primer extension and S1 nuclease digestion of hPL cDNA-placental poly(A+) RNA hybrids. All three methods showed the ratio of hPL_4 to hPL_3 mRNA was 3:2. As these experiments reflected RNA levels from a single term placenta, hPL RNA from a further ten placentae were subjected to S1 nuclease analysis with the determination that there was wide variability in the ratio of hPL_4:hPL_3 mRNA (S.L.F. and G.F.S., unpublished data).

Regulation of hPL_3 and hPL_4 expression could be explained by sequences in the promoter that regulate transcriptional initiation or differences in the transcribed sequence that could contribute to mRNA stability. The latter theory was tested by placing each structural gene under the control of the SV40 enhancer and metallothionein promoter and transfecting COS-7 cells. Slightly more protein was produced from the hPL_3 gene construct than the

hPL_4 constuct (65). To determine the cause of such heterogeneity, the first two exons of the hPL and hGH genes were exchanged. Five-fold chimeric protein and eight-fold less mRNA was present when the first two exons of the hPL_4 were present than when the first two exons were derived from hPL_3 (Fig. 4). Protein levels resulting from the first two exons of hGH and the last three exons of hPL_4 were comparable to intact hGH, suggesting that only the first two exons contribute to differential expression. The greater stabilty of hPL_3 versus hPL_4 mRNA counters the greater expression hPL_4 mRNA *in vivo* and therefore may contribute to the variable levels of hPL_3 and hPL_4 mRNA in placentae.

The hPL_1 transcript was not detected in the original cDNA cloning (2) and lack of expression was attributed to a mutant RNA splicing site (43). Surprisingly, the hPL_1 transcript was detected by Chen *et al.* (54) in a placental cDNA library at an abundance of 0.01% using oligonucleotides specific for hPL_1, however, nine of ten cDNA clones analyzed were derived from incompletely processed hnRNAs and the other clone was found to have used an alternative splice site to overcome the mutation in the second intron splice donor site. Therefore, it is still uncertain whether hPL_1 can produce a viable protein *in vivo*. In order to determine if the aberrant splice site was wholely responsible for the lack of hPL_1 expression, a region of the hPL_1 cDNA encoding the second exon/intron boundary was exchanged with the functional equivalent region of hPL_3 (65). No protein or mRNA was produced from the

mutated hPL_3 gene or the "repaired" hPL_4 gene. The lack of function of the "repaired" hPL_4 gene is not yet understood but suggests other nucleotide differences must contribute to the lack of hPL_4 expression.

Regulation of hPL and hGH Gene Expression

The similarity of the hPL and hGH genes at the nucleotide level is striking as the hGH-N, hPL_3, and hPL_4 genes have 92.5% sequence identity through their exons, with the 464 bp 5' to the initiation site having 93.8% homology (50). The highly homologous nature of the hPL and hGH genes and 5' flanking regions suggest that very precise mechanisms regulate their tissue specific expression.

Understanding the regulation of hPL gene transcription has been facilitated by studies of hGH-N gene regulation. The hGH-N gene has been well characterized in terms of DNA sequences and transcription factors that regulate expression. It is known that 5' flanking sequences within 289 bp of the cap site mediate hGH tissue specific expression and that binding of the pituitary specific factor GHF-1 in this region permits transcription of hGH in the pituitary (66-68). GHF-1 is a 33 kD polypeptide (69) known to be closely related or identical to the Pit-1 protein that binds and regulates the rat growth hormone and prolactin gene promoters (70). Analysis of cDNA clones encoding GHF-1 indicates that it is a homeobox containing protein. GHF-1 binds the hGH promoter at two sites: -55 bp to -90 bp and -106 bp to -131 bp (68-71). In

addition to GHF-1, other general transcription factors bind to the hGH promoter (71). The Sp1 transcription factor binds nucleotides -131 to -140 adjacent to the distal GHF-1 binding site. Further upstream the USF/MLTF protein binds the region -237 bp to -267 bp, adjacent to the binding site of an unknown protein at -267 bp to -290 bp. Comparison of DNase I footprints showed the pattern of protein binding for hGH-N and hPL_3 promoters is nearly identical (72). Further studies show the pituitary specific factor GHF-1 is able to bind to the hPL promoter (71); however, this may not be significant as no GHF-1 is believed to be present in the placenta (67).

Thyroid hormone can also bind to a region of the hPL promoter in a DNA-binding assay (73). When introduced into a rat pituitary cell line, thyroid hormone and dexamethasone could increase expression of a transient reporter gene containing 500 bp of the hPL promoter (74). Therefore the hPL promoter may be regulated by dexamethasone and thyroid hormone. The physiological significance of these studies is unclear since pituitary and not placental extracts or cell lines were used.

DNA Sequences in the hPL_3 gene promoter necessary for transcriptinal activity.

The initiation sites of hPL_3 and hPL_4 gene transcription have been studied by two groups (50, 75). Both studies found 82-95% of transcripts initiate 30 nucleotides (nt) downstream from a TATA sequence (and 63 nt upstream from the AUG and start of translation). An additional initiation region 23 nt upstream of

the TATA sequence and 30 nt downstream from a CATAAA sequence was found to be responsible for 5-8% of hPL transcripts. Interestingly, the hPL_1 gene promoter was shown to be functional and acted similarity to hPL_3 and hPL_4 promoters in a cell-free transcription system (75).

More recent work has concentrated on the promoter and an enhancer of the hPL_3 gene (Fig. 5). Deletion mutants of the hPL_3 promoter were analyzed for transcriptional activity following transient transfection of a placental cell line (76). Various regions of the hPL_3 promoter extending from -1200 to -77 bp were inserted in a plasmid vector adjacent to the bacterial gene for chloramphenicol acetyl transferase (CAT). The vector also contained the hPL_3 enhancer (discussed below) to increase the level of transcription. The plasmids were transfected into JEG-3 cells, a hPL-producing placental choriocarcinoma cell line (77), with the resulting CAT activity reflecting the transcriptional activity of the hPL_3 promoter. These studies (Fig. 6) indicated sequences between -142 bp and -129 bp were important for hPL_3 promoter activity since removal of this region reduced CAT activity 8-fold. Internal deletions of this region (AR, SA, Fig. 6) confirm those seen using 5' deletions. Similar results (S.L.F. and G.F.S., unpublished data) were seen using JAR cells, another human placental trophoblast cell line (78). No other region in the promoter (within 1 kb 5' of the hPL_3 gene) was found to be important for induction of transcriptional activity in placentally derived cells.

The hPL_3 promoter regulatory sequence (-142 bp to -129 bp) does not appear to regulate tissue-specific expression based on functional and binding studies. In the presence of the hPL_3 enhancer, the hPL_3 promoter (-500/+2 bp) was able to activate CAT transcription in JEG-3 cells but not in HepG2 cells, a human liver cell line. However, paired with the SV40 enhancer, the hPL_3 promoter directed gene expression in a non-tissue specific manner as transfected JEG-3 and HepG2 cells produced similar levels of CAT activity. In these studies transcription in JEG-3 and HepG2 cells not only required the appropriate enhancer but also the hPL_3 promoter regulatory region (-142 bp to -129 bp). Without this sequence, only background levels of CAT activity were observed. This suggested that the hPL_3 enhancer was responsible for tissue-specific gene expression whereas the hPL_3 promoter regulatory sequence (-142 bp to -129 bp) was required for basal expression. While upstream sequences within 1200 bp of the hPL_3 gene did not direct transcription in a tissue-specific manner, there may be additional tissue-specific sequences further upstream. All placentally expressed members of the hPL/hGH gene family contain a 1 kb region of high homology located 2-3 kb 5' to the start of transcription (54) but the functional significance of this region remains to be determined.

The hPL_3 promoter regulatory sequence (-142 bp to -129 bp) binds specifically nuclear proteins from JEG-3, HepG2, and HeLa cells as shown by gel shift assay (76), again suggesting that this sequence is important for basal gene activity but does not play a

role in tissue-specific gene expression. This region of the promoter contains the sequence GGGAGG, which is a variant of the binding site GCGCGG for the ubiquitous transcription factor Sp1 (79). This variant Sp1 binding sequence, which binds Sp1, is also present in promoters of other genes including HIV LTR (80-82) rat insulin-like growth factor II (83), and human hsp 70 (84). Gel shift assays demonstrated that the addition of DNA containing the Sp1 binding site specifically competes for proteins binding the the hPL_3 promoter regulatory sequence. Further competition studies employing a variant Sp1 site GGGGG, which is not recognized by Sp1 (85), showed that this oligomer did not compete for JEG-3 proteins binding to the hPL_3 promoter regulatory region. The binding specificity of the proteins interacting with the hPL_3 promoter regulatory region was further characterized by determining nucleotides of the hPL promoter regulatory sequence interacting with DNA-binding proteins using a methylation interference assay. The methylation interference pattern of placental nuclear proteins binding to the hPL_3 regulatory sequence was identical to that shown previously for Sp1 binding to its binding sites. These results suggest that the hPL_3 regulatory region binds Sp1 or an Sp1-like protein which is necessary for basal transcriptional activity.

Sp1 regulates transcription of many different kinds of genes (79). However it does not directly interact with RNA polymerase (86) or the transcription factor TFIID, which binds the TATA sequence (87, 88), suggesting that Sp1 requires accessory factor(s) or coactivators to activate transcription (88). One such factor

could include the protein(s) binding the tissue specific hPL_3 enhancer.

An Enhancer 3' of the hPL_3 Gene Stimulates Tissue-Specific Transcription.

In order to explain the tissue specific expression of the hPL_3 genes Rogers *et al.* (89, 90) conducted a search of the entire hPL/hGH gene cluster for the presence of transcriptional enhancers. A series of *Eco*R1 fragments covering nearly the entire 66 kb gene cluster including the five genes and their flanking regions were tested for enhancer activity. Only one *Eco*R1 fragment, was found to have enhancer activity in JEG-3 cells. This 3.9 kb fragment was located 3' to the hPL_3 gene at the distal end of the hPL/hGH gene cluster (Fig. 7). This fragment fulfilled all the required definitions of an enhancer as it was active either 5' or 3' of a gene and in either orientation and was able to activate a heterologous promoter.

Further analysis of the hPL enhancer localized enhancer activity to a 1 kb *Acc*I - *Sac*I restriction fragment located 2 kb 3' of the hPL_3 gene. The 1 kb enhancer was shown to have great tissue preference in its action as the enhancer was 20-fold more active in human trophoblast JEG-3 cells than the rat pituitary cell line 18-54-SF (89). Therefore, the hPL enhancer is likely to be the element responsible for the tissue specific-expression of the hPL_3 gene.

Further transient transfection experiments employing JEG-3 and JAR cell lines allowed localization of the hPL enhancer to a 138 bp region located between nucleotides 103-241 of the 1 kb *Acc*I - *Sac*I restriction fragment (Fig 8) (91). The 138 bp hPL enhancer when linked 3' to the CAT gene increased CAT expression 25 to 30-fold over that due to the SV40 promoter alone. The 138 bp region also contained all information necessary to impart tissue-specific gene expression as plasmid constructs containing this region are 20 to 40-fold more active in JEG-3 cells than non-hPL producing human HepG2 liver, U-373 MG glioblastoma and, HeLa epithelial cell lines. Additional studies support the idea that the hPL enhancer may be the major determinant of tissue-specific expression of the hPL_3 gene. As stated previously, Fitzpatrick *et al.* (76) have shown that hPL_3 promoter fragments extending to -496 bp upstream of the gene had similar activities in JEG-3 and HepG2 cells. Therefore, the hPL enhancer is the major positive tissue specific regulator of hPL_3 gene transcription.

Enhancer-Nuclear Protein Interactions

A 210 bp fragment (1-210) containing all the information necessary for tissue-specific enhancer function was found to specfically interact with nuclear proteins from placenta tissue, JEG-3 and JAR cells. Band retardation assays showed that up to three specific protein-enhancer complexes are formed with protein extracts from placental lactogen-producing cells. These complexes

are identical for all the extracts from hPL-producing cells. However, protein-DNA complexes of different electrophoretic mobilities and affinities were formed following the incubation of HeLa and HepG2 nuclear proteins with the 1 - 210 bp enhancer fragment.

DNase I protection experiments showed that proteins from both placental and HeLa cell nuclear extracts protect a 22- bp region (115 - 137) of the hPL enhancer encompassing a TEF-1 motif (TGGAATGTG) located at positions 126 - 133 (Fig. 9). The TEF-1 motif was first identified by Xiao *et al.* (92) in studies of the SV40 enhancer and has been shown to bind a 53 kDa protein called TEF-1 (93). Binding of this protein to the TEF-1 motif correlates with *in vivo* SV40 enhancer activity (92). TEF-1 protein is found in cell types (HeLa and undifferentiated and differentiated F9 embryonal carcinoma) other than those that produce hPL. However, TEF-1 shows some tissue specificity, as no TEF-1 activity is found in lymphoid cell lines (92, 93). The TEF-1 protein binds cooperatively to tandem repeats of the TEF-1 motif or to closely linked *Sph*I (AAGCATGCA) or *Sph*II (AAGTATGCA) motifs. When present in tandem repeats or when associated with Sph motifs, TEF-1 is a strong transcriptional activator.

The region of DNase I protection (115-137) seen over the TEF-1 motif using the hPL enhancer is similar to that reported in studies of the TEF-1 motif in the SV40 enhancer (93). Though these two enhancers have no sequence similarity except for the nine

nucleotide TEF-1 site, a 22-nucleotide region of the hPL enhancer encompassing the TEF-1 motif is protected by placental and HeLa extracts, whereas a 21-nucleotide region of the SV40 enhancer is protected by HeLa nuclear extracts. This suggests a protein similar to TEF-1 binds the hPL enhancer. However, band retardation experiments using the hPL enhancer showed that proteins from HeLa and placental extracts have different affinities for the 1-210 bp enhancer fragment and the protein-DNA complexes formed due to these proteins have dissimilar mobilities in polyacrylamide gels (91). Also, placental and HeLa nuclear proteins cause different patterns of DNase I hypersensitivity, suggesting their binding alters DNA conformation in different ways. This difference in binding characteristics of placental and HeLa enhancer binding proteins may reflect different protein modifications, a family of TEF-1 like proteins with individual members expressed in specific tissues, or different regulatory proteins that bind to similar DNA recognition sites.

Regions 3' of the hPL_1 and hPL_4 Genes are Homologous to the hPL_3 Gene Enhancer

Inspection of the hPL/hGH gene cluster DNA sequence revealed the presence of two other putative enhancers located 3' to the hPL_1 and hPL_4 genes (91). The enhancers are located in the same relative location as the hPL_3 enhancer, approximately 2.2 kb 3' to their respective genes. These regions are 95% homologous with the 1022 bp hPL_3 enhancer and the region shown to interact with placental nuclear protein(s) by DNase I footprinting is exactly

duplicated in both the hPL_1 and hPL_4 flanking regions (Fig. 10). No homology to the hPL_3 enhancer was detected anywhere else in the 66 kb hPL/hGH gene cluster including hGH-N or hGH-V flanking regions.

An explanation for the limitation of enhancers to the 3' flanking regions of the hPL genes has been proposed (42). It is postulated that the 3' breakpoint of the original duplication event leading to the precursor hPL and hGH genes occurred within an *Alu* repeat about 100 bp downstream from the hPL-hGH ancestral gene. It is thought that the duplication event most likely involved a homologous, unequal crossover between this *Alu* repeat and another found 8 kb upstream of the hGH_1 gene (see Figure 3). The major significance of the initial duplication event is that it would cause the 3' regions close to the hPL and hGH genes to be unrelated. This would account for the presence of a placenta-specific regulatory element 3' of hPL but not hGH genes. Also the presence of an enhancer 3' of the hPL_4 gene would explain why hPL_4 mRNA was found to be at least as abundant or more abundant than hPL_3 mRNA (2, 54) while lack of enhancer homology near hGH genes correlates with low placental hGH expression.

FIGURE LEGENDS

Fig. 1. The primary amino acid structure of human placental lactogen. The two disulfide bridges are marked by heavy shading. Reproduced with permission from Li, 1972 (1).

Fig. 2. Physiological role of human placental lactogen during pregnancy. The current model of the functional role of hPL in maternal metabolism is, by its lipolytic and insulin antagonist activity, to increase preferentially glucose availability for the fetus. (-) = inhibitory effect. Reproduced with permission from Yen, 1989 (94).

Fig. 3. Schematic diagram of the evolution of the hGH/hPL gene family redrawn with permission from Hirt *et al.*, 1987 (43). Gene sequences are indicated by stippled boxes, *Alu* sequences noted by solid boxes and partial *Alu* sequences noted by open boxes. The latter indicate differences between the 3' ends of the hPL and hGH genes. The present day gene family is believed to have originated from a single ancestral gene by three duplication and nonreciprocal crossover events ((X)).

Fig. 4. Diversity in size and abundance of protein expression products from hPL and recombined hPL/hGH genes. Reproduced with permission from Reséndez-Pérez, 1990 (65). A. Map of the hybrid

genes which are composed of hPL-3 (solid) or hPL-4 (hatched) and hGH (open) gene portions. B. Proteins produced from COS-7 cells transfected with hPL/hGH hybrid genes. As a negative control lane (-), COS-7 cells were transfected with a vector lacking hPL and hGH gene sequences. Intact hGH genes produce 20 kD and 22 kD proteins while the hPL protein is 25 kD. C. Slot Blot analysis of hPL-3 and hPL-4 RNA expression. Total cellular RNA was obtained from COS-7 cells transfected with a vector containing either the hPL-3 or hPL-4 genes. RNA was applied to each slot of a slot blot apparatus and the filter hybridized to an hPL cDNA probe and a DHFR cDNA probe.

Fig. 5. Restriction map of the hPL_3 gene and flanking region. Promoter deletion mutants were constructed from the 500-bp *Eco*R1 (-497 bp)/*Bam*HI (+2 bp) sequence. An enhancer is located 2.2 kb 3' to the gene within the *Acc*I-*Sac*I restriction fragment. Open boxes, Human PL_3 exons. Restriction enzyme cleavage site abbreviations are as follows: AI, *Ava*I; AII, *Ava*II; Ac, *Acc*I; Au, *Alu*I; B, *Bam*HI; D, *Dde*I; E, *Eco*RI; H, *Hinf*I; P, *Pvu*II; Ps, *Pst*I; S, *Sac*I; Sa, *Sau*3A; St, *Stu*I; X, *Xba*I. Reproduced with permission from Fitzpatrick, 1990 (76).

Fig. 6. Transient expression of hPL_3 CAT deletion mutants in JEG-3 cells. (A) Deletion mutants of the hPl_3 promoter with varying amounts of 5' sequence (relative to the start of transcription)

were ligated 5' to a promoterless bacterial gene CAT. Three internal deletion mutants, -232 AR (Δ-129/-77), -232 MR (Δ-142/-77), -390 SA (Δ-152/-129), were also constructed. Horizontal bars indicate DNA sequences included in each construct. The hPL_3-CAT DNA was cotransfected with a reporter plasmid containing the β-gal gene into JEG-3 cells, a human placental choriocarcinoma cell line. CAT activity was expressed relative to β-gal activity as the mean ± SD. (B) JEG-3 cells were transfected with plasmids containing the hPL_3 promoter mutants described in A, and the CAT activity is shown by the autoradiograph. AR, MR, and SA refer to internal deletion mutants (A). Positive (pSV2CAT) and negative (pCAT3M; mock) controls were included. Reproduced with permission from Fitzpatrick, 1990 (76).

Fig. 7. Restriction map of the human placental lactogen and growth hormone multigene cluster. The map and gene nomenclature is a composite from maps previously published. The structural gene regions are indicated by the open blocks and the direction of transcription is from left to right. Each of the vertical bars represent an *Eco*RI restriction site. Reproduced with permission from Rogers and Saunders, 1986 (90).

Fig. 8. Activity of hPL_3 enhancer deletion mutants in JEG-3 cells. JEG-3 cells were transfected with an SV40-enhancer containing vector (pSV2CAT), enhancerless vector (pSV1ϕAT), or pSV1CAT

constructs containing various deletion mutants of the 1022 bp *Acc*I-*Sac*I hPL_3 enhancer. Activity of each construct is normalized to activity of pSV2CAT = 100%. Horizontal bars refer to the region of *Acc*I-*Sac*I fragment remaining in constructs. Reproduced with permission from Walker *et al.* 1990, (91).

Fig. 9. Interaction between placental nuclear proteins and DNA sequences in the hPL_3 enhancer. A DNA fragment containing the hPL_3 enhancer was incubated with a placental nuclear extract and digested with DNAse I. The region protected from enzyme digestion is shown by the brackets.

Fig. 10. Comparison of hPL_2 and hPL_1 3' flanking regions with the hPL_3 enhancer. Slashes represent gaps in nucleotide homology. Underlined nucleotides signify the TEF-1 motif.

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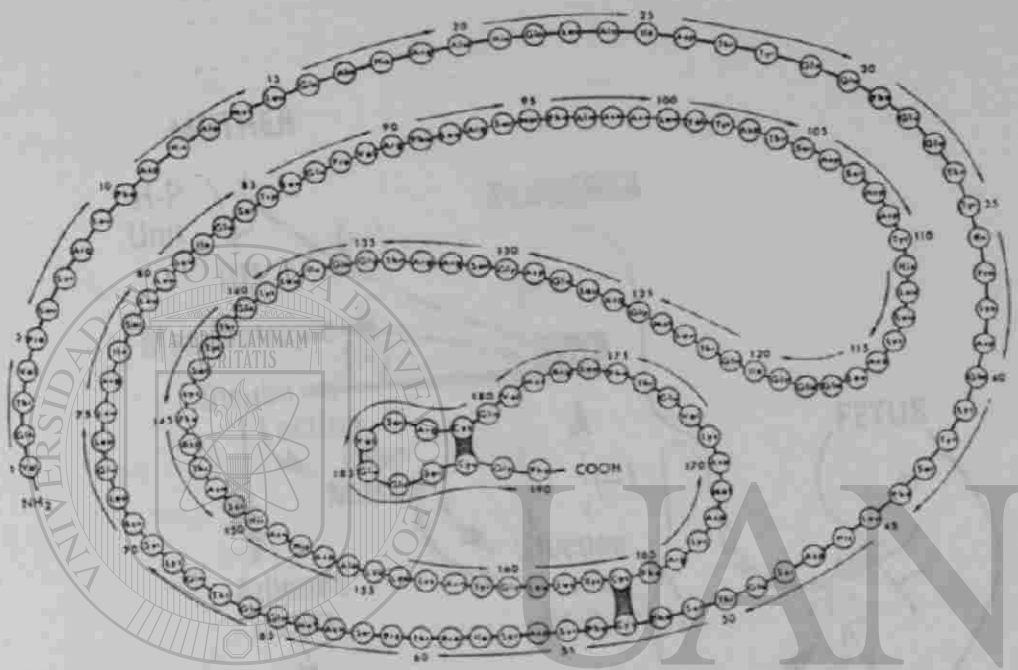

Figure 1

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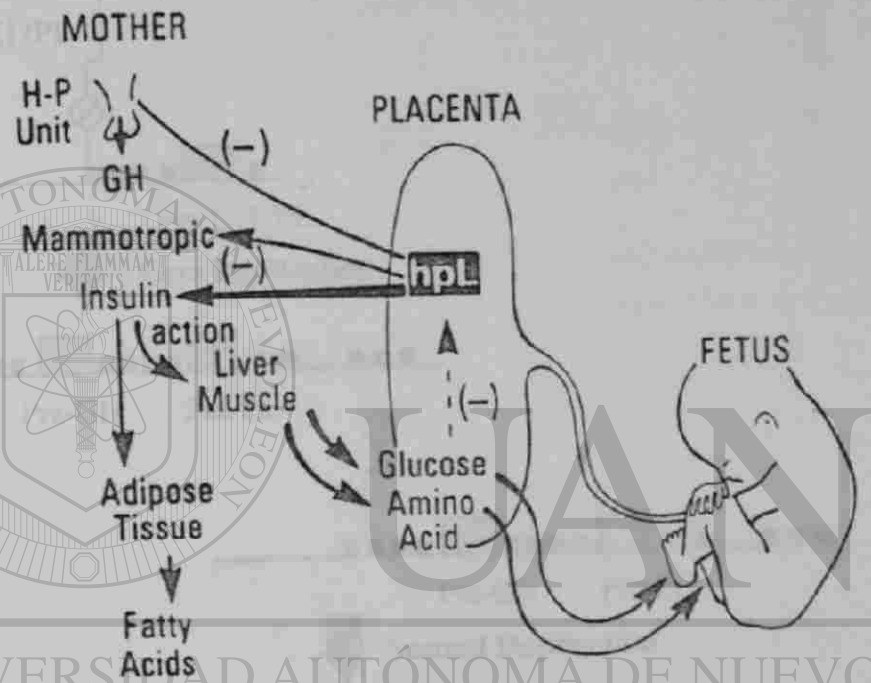
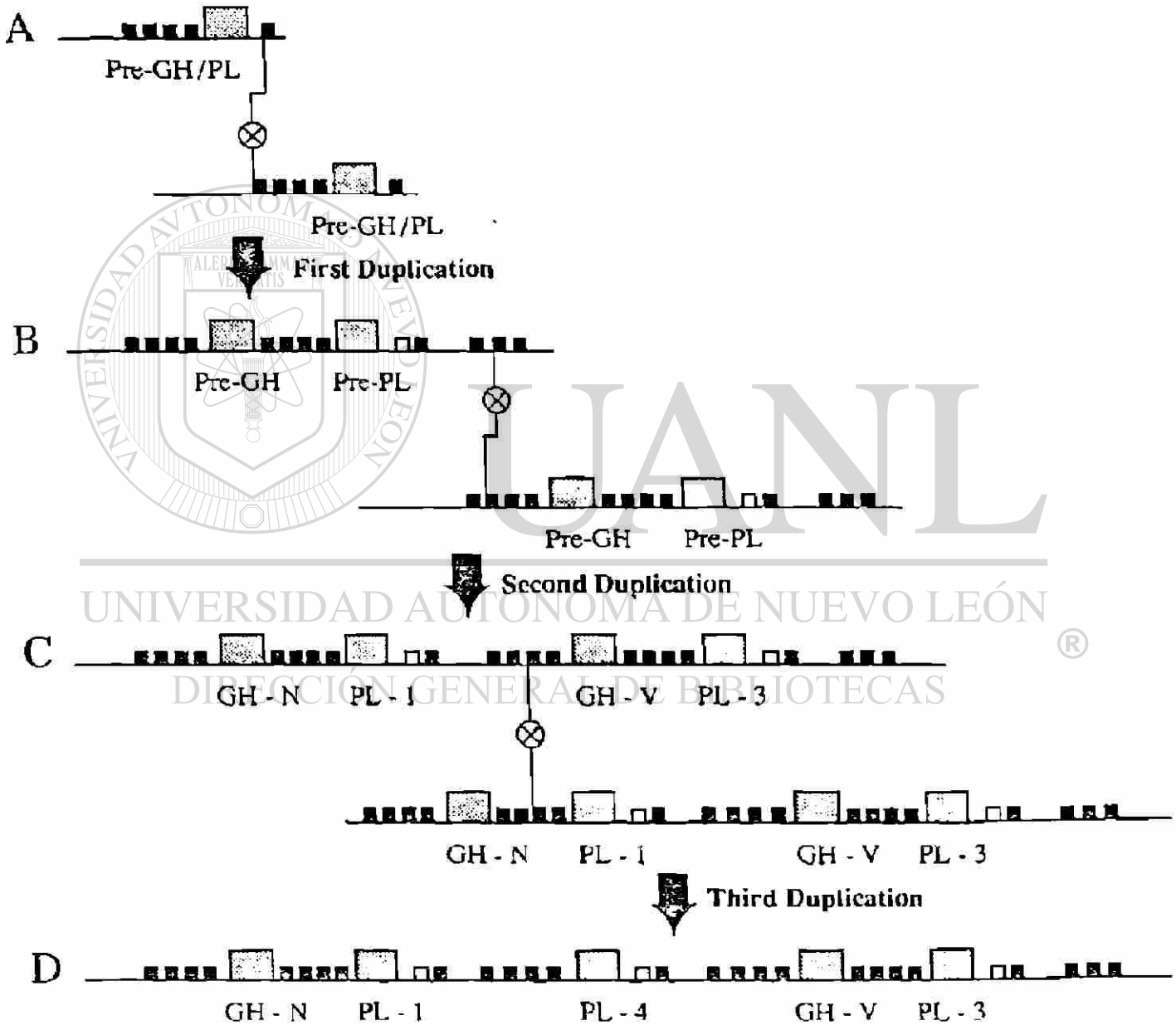


Figure 2

Evolution of the Growth Hormone / Placental Lactogen Gene Family



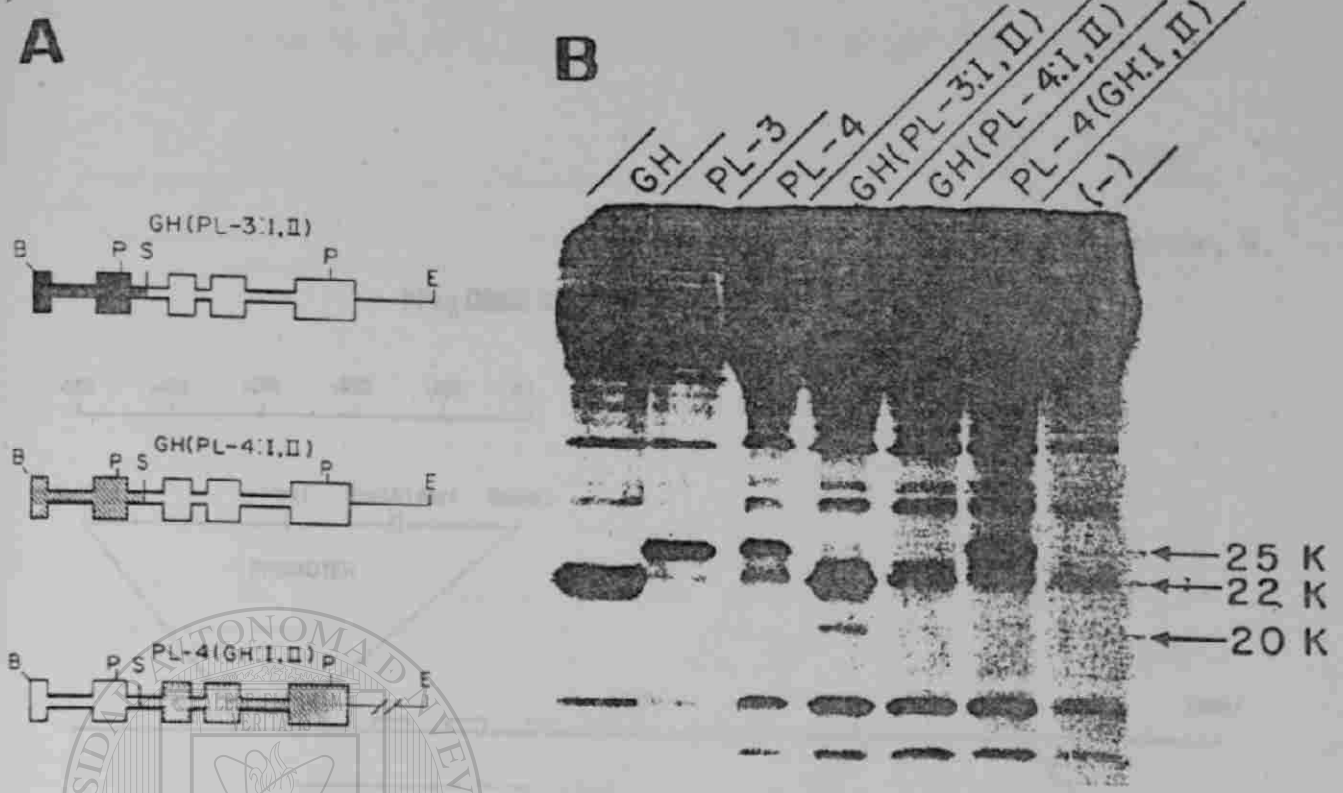


Figure 4A,B

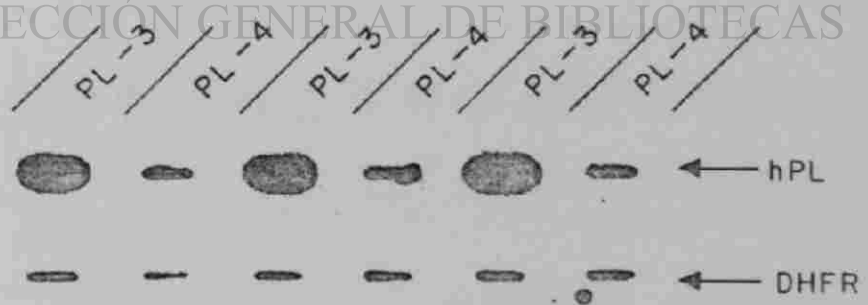


Figure 4C

hPL_3 GENE AND REGULATORY SEQUENCES

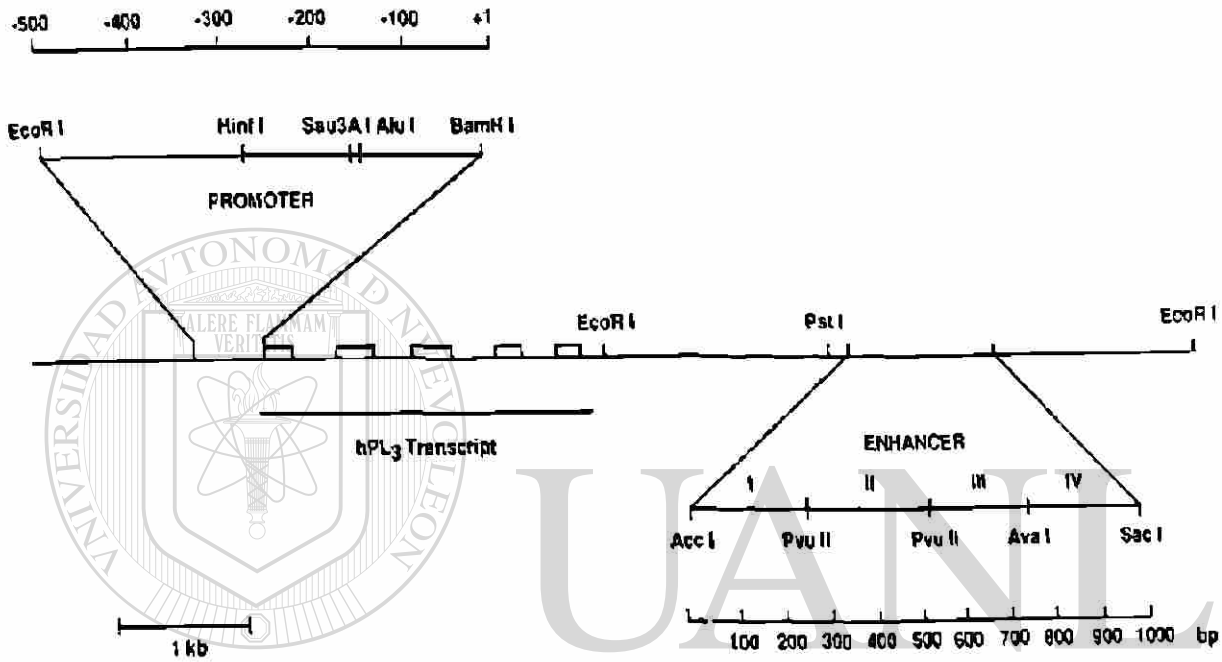


Figure 5

Deletion mutants of hPL promoter and CAT activity in JEG-3 cells

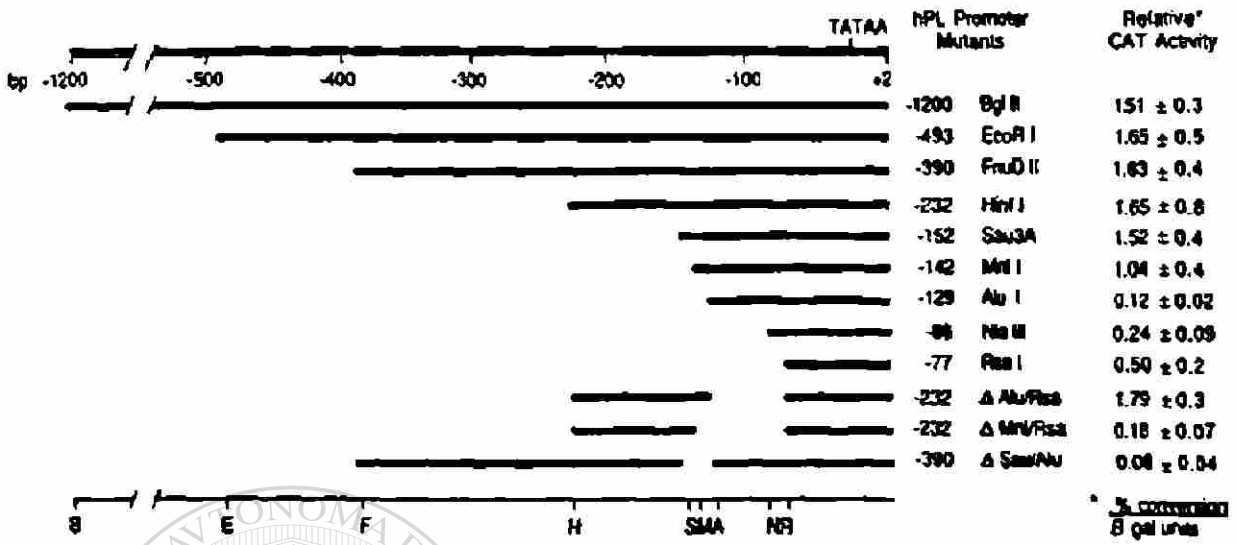


Figure 6A

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Activity of hPL Promoter Deletion Mutants in JEG-3 Cells

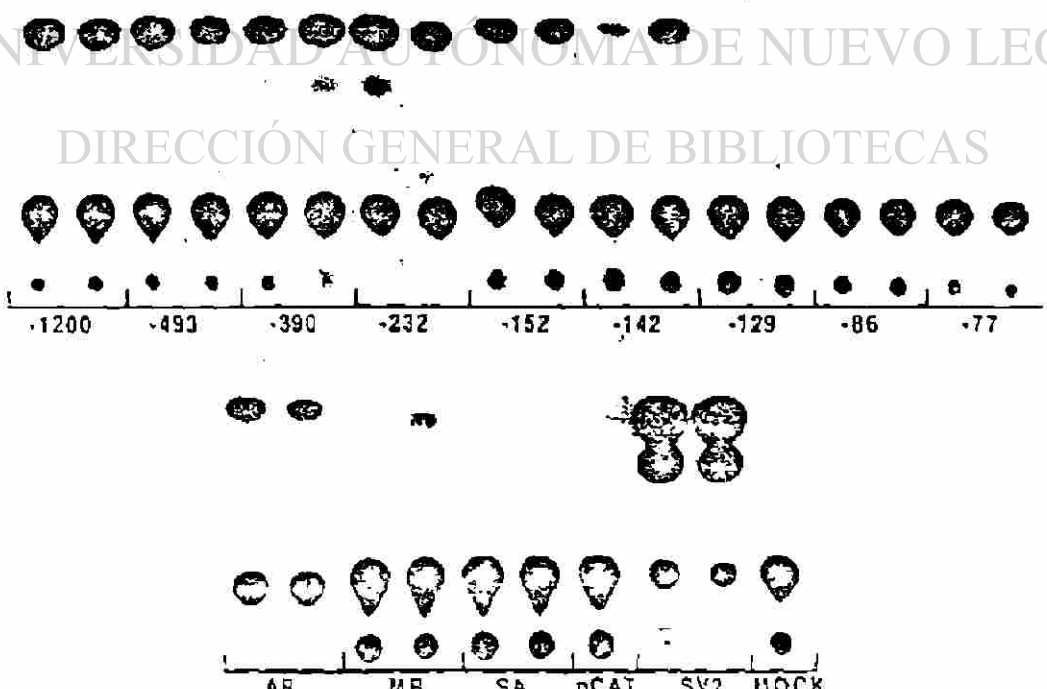

Figure 6B

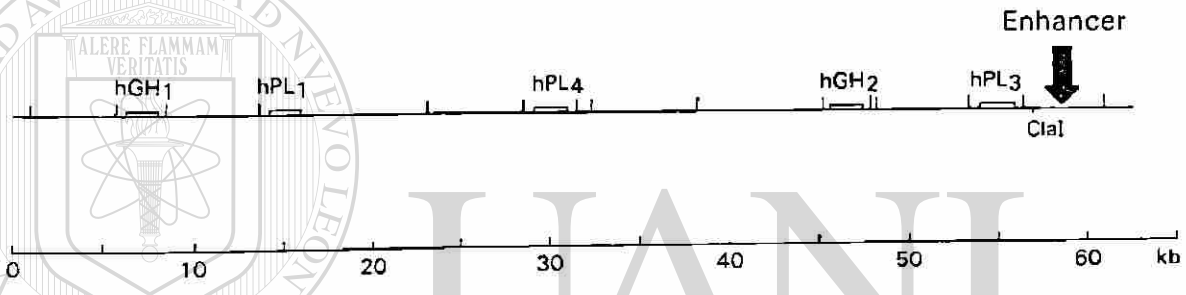

Figure 7

Summary of hPL Enhancer Deletion Mutants Activity in JEG-3 Cells

Construct	% pSV2CAT	S.D.	Ob
1) 1-1022	84	14	11
2) 1-730	45	11	7
3) 158-1022	0.7	0.1	7
4) 289-932	1.6	1.4	5
5) 1-134	2.0	1.4	6
6) 1-210 (+)	79	17	7
7) 1-210 (-)	72	9.8	6
8) 103-404	87	33	9
9) 103-241 (+)	78	24	4
10) 103-241 (-)	61	16	4
11) Δ241-506	40	16	8
12) 241-506	1.2	0.1	6
13) 210-404	2.0	1.0	6
14) pSV1CAT	1.2	1.2	6

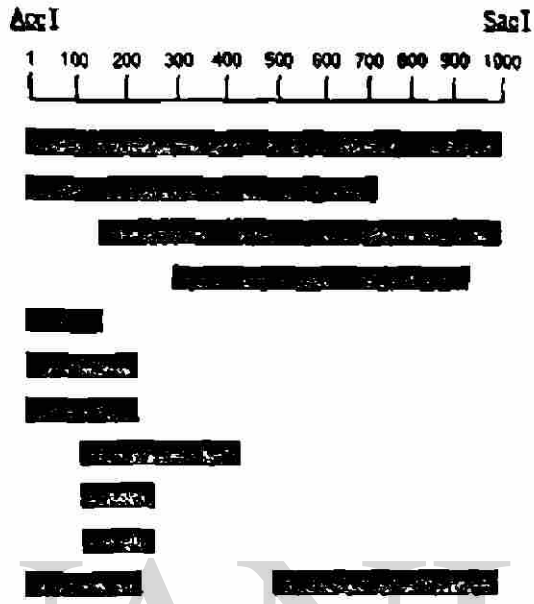

Figure 8

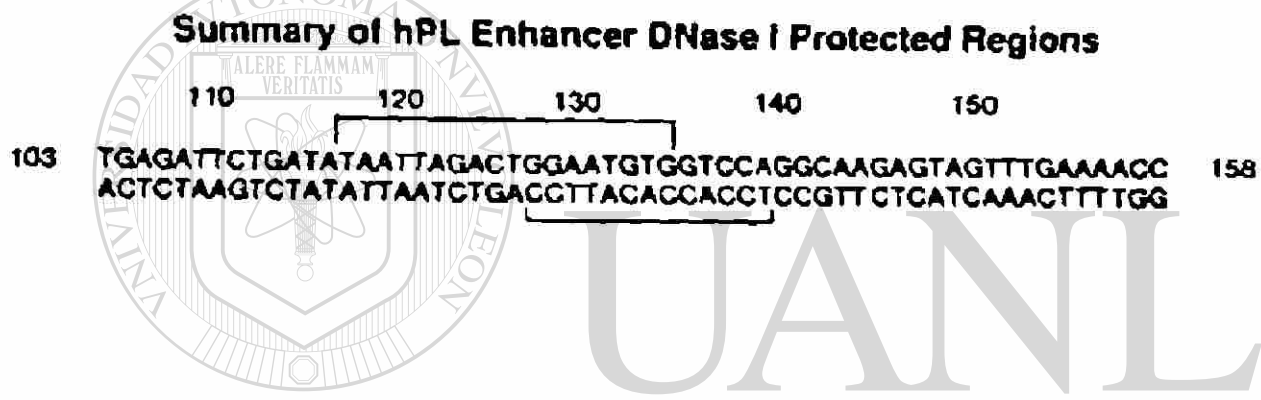

Figure 9

Homology of hPL_3, hPL_1 and, hPL_4 3' Flanking Regions

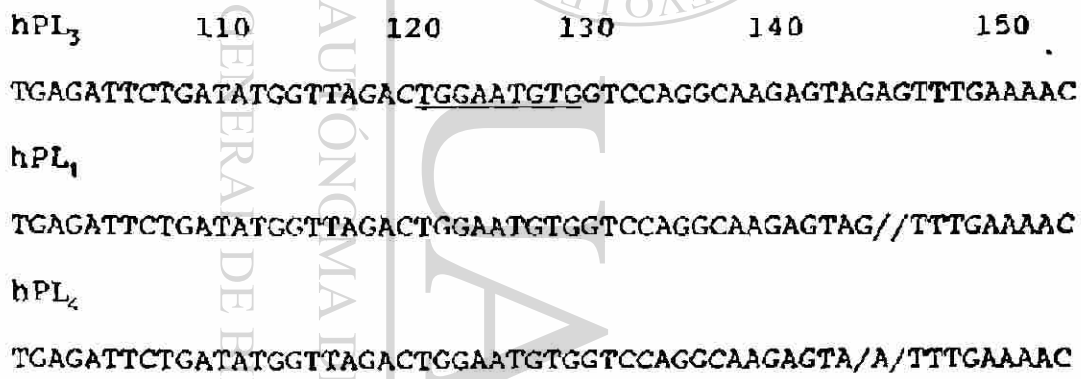

Figure 10

ANEXO C

xpression Studies of ransfected Multigene amilies by Homologous NA Mutagenesis

BSTRACT

A valuable approach for multigene mily studies where the expression oduct of at least one gene member of e family is measurable is described. In ch cases, the effect on gene expression nucleotide sequence differences or utations occurring in other members of e family or at alleles can easily be deter-ined. This is achieved by a strategy lled homologous DNA mutagenesis. It nsists of the insertion of mutated gions from homologous genes into the ntext of the gene coding for the assay-ble product. Here we demonstrate the asibility of this approach using gene embers of the human growth hormone nd human placental lactogen (hGH-hPL) ultigene family.

NTRODUCTION

Advances in recombinant DNA echnology have made the task of solating genes from higher eukaryotes asier. However, many of the protein-ncoding-type genes exist in the mam-nalian genome as families with multi-le non-allelic copies with very similar sequences. An approach to studying multigene families has been to exten-sively examine one member and then predict the nature of its expressed protein and those expressed by its re-lated members. Nevertheless, proving that all members of the multigene fami-ly are expressed can be a formidable task.

Several strategies have been em-ployed to achieve this goal. For ex-ample, if the presence of specific mRNAs on a polysome fraction can be demonstrated, then it may be suggested that translation occurs and that the genes are being expressed (7). In other cases, the use of mRNA complemen-tary DNA (cDNA) cloning in conjunc-tion with prokaryotic expression vectors permits the generation of anti-bodies. These are raised against fusion proteins expressed by the vector along with the cDNA coding protein (3). The antibodies then can be used to inves-tigate the *in vivo* temporal and cellular parameters for the expression of these genes.

In studies of gene expression, it is ideal to be able to directly characterize the expressed protein. However, to quantitatively discriminate from at least 50 000 other proteins of the eukaryotic cell, the vector's expression control signals must be strong and, pref-erably, should be of wide tissue spec-ificity. There are several vectors that fulfill these criteria and take advantage of the use of transcriptional control sig-nals from mammalian viruses (5).

Mutations can affect expression levels in eukaryotic cells due to an aberration in any one of the many steps of the gene expression pathway: tran-scription, RNA processing, mRNA stability or translation (15). The effects of single base mutations in the expres-sion of structural sequences are fre-quently studied but require a more selective, analytical method of assess-ing such changes.

Here we describe a strategy, called homologous DNA mutagenesis, to ac-curately evaluate the effect of small mutations occurring at pseudogenes, functional non-allelic copies or mutat-ed alleles of multigene families. It con-sists of the introduction of the mutated area, with a minimal amount of flank-ing DNA sequences, into the structural region encoding a homologous so-called reporter gene for which anti-bodies or a quantitative assay are avail-able. We used this system to examine the influence on gene expression of se-quence variations found in hPL genes. In addition, we inserted a mutation oc-curring at a putative hPL pseudogene (1) into the context of the hGH struc-tural gene. The convenience of using hGH as a reporter protein (13) allowed us to use a simple radioimmunoassay to quantify the effect of these nucleo-tide sequence changes.

MATERIALS AND METHODS

Construction of Recombinant Plas-mids and Preparation of Their DNA

Restriction and other enzymes were obtained from commercial suppliers (BRL/Life Technologies, Gaithers-burg, MD, and New England BioLabs, Beverly, MA) and used according to the manufacturers' instructions. The characterization of hPL genes has al-ready been described (1). The vector described here was derived from pNUT (9), a gift from Richard Palmiter, and consisted of the larger DNA fragment resulting from *Bam*HI plus *Eco*RI digestion of this plasmid. This frag-ment was isolated (14) and ligated to the DNA fragments, flanked by the same sites, carrying the gene fragments of interest. Ligations and transforma-tions were carried out as previously described (2,8). Recombinant plasmids were first isolated from 3-ml overnight cultures and then characterized by restriction analysis, followed by large-scale preparation (8). pMThGH111 (10) was also a gift from Richard Pal-miter, and pSV2gpthGH (12) was a gift from Grady Saunders. pCMVhGH was constructed in our laboratory by replac-ing the chloramphenicol acetyltrans-ferase (CAT) sequences in pCMVcat (4) with structural hGH gene.

Cell Culture, DNA Transfection and Detection of Transiently Expressed Proteins

COS-7 cells (a gift from Tien Kuo) were adapted to grow in Dulbecco's modified Eagle's medium (Sigma Chemical, St. Louis, MO) containing 1% fetal calf serum (FCS) (Hyclone Laboratories, Logan, UT). They were maintained at 37°C with 5% CO_2. Plasmid DNA (7.5 μg/60-mm-diameter Petri dish) was transfected by the cal-cium phosphate method (6). Efficiency of transfections was normalized by co-transfecting with pCMVcat and moni-toring CAT activity (5).

Quantification of hGH and hPL-hGH chimeric proteins was achieved using a commercially available radio-immunoassay kit (Diagnostic Products, Los Angeles, CA). Media from cul-tured cells were diluted and assayed using the protocol included with the kit and briefly summarized as follows: 0.1 ml of calibrators (ranging from 0–30 ng/ml, the latter concentration cor-responding to 60 μIU/ml of the World Health Organization standard) and diluted samples were added to 12 × 75-mm tubes. Anti-hGH rabbit serum (0.1 ml) was added to the tubes and the mix-

tures were incubated for 1 h at room temperature. This antibody is known to be highly specific for hGH. It presents cross-reactivities to hPL, thyroid stimulating hormone (TSH), follicle stimulated hormone (FSH), luteinizing hormone (LH) and towards a subunit β of human chorionic gonadotropin (HCG) of less than 0.006%. To each tube was added 0.1 ml of ^{125}I-hGH and incubation continued for another hour. A second antibody (goat anti-rabbit serum) (1.0 ml) mixed with PEG in saline solution was added. After centrifugation, the supernatant was decanted, and the radioactivity remaining in the tubes was determined. Cpm values of experimental samples were used to estimate hGH concentrations using the known hGH concentrations of the calibrators as reference.

RESULTS AND DISCUSSION

Choice of Expression Vector

The radioimmunoassay values of secreted hGH from DNA transfection experiments were compared for plasmid constructs pSVgpthGH, pMThGH, pCMVhGH and pNUT. As illustrated in Figure 1, in plasmid pSVgpthGH, the hGH structural gene is under the control of natural hGH gene promoter and a copy of the simian virus 40 (SV40) enhancer. The second construct, pMThGH, has the hGH structural gene joined to the metallothionein promoter. The third construct, pCMVhGH, has the cytomegalovirus enhancer-promoter pair driving the expression of the hGH structural gene (11). Finally, pNUT uses the metallothionein promoter and SV40 enhancer to express the promoterless hGH-N gene. The level of hGH secreted into the medium of cells transfected in parallel with each of these plasmid constructs was found to be highest with pNUT (data not shown); therefore, we chose pNUT for the rest of the study.

Table 1. Transient Expression[a] of Human Growth Hormone Secreted Into the Media of Transfected COS-7 Cells

Gene	hGH Production[b]
None	0.3 ± 0.73
hGH-N	656.03 ± 126.32
GH(PL-3:I,II)	408.68 ± 69.79
GH(PL-3m:I,II)	0.28 ± 0.68
GH(PL-4:I,II)	82.94 ± 30.31

[a] Plasmids transfected consisted of the pNUT vector described in Materials and Methods, carrying as insert the gene indicated.

[b] Values, average and standard deviations, are in ng of hGH/culture dish, obtained by radioimmunoassay. Each represents data from three independent cotransfection experiments using a different set of plasmid DNA preparation and pCMVcat to compensate for variations in transfection efficiency.

Homologous DNA Mutagenesis

We chose to examine the effects of two different types of mutations on gene expression. The first being that of sequence variations among the first two exons of the hPL-3 and hPL-4 genes. The second corresponding to an important donor splice point mutation (GT→AT) found at the beginning of the second intron of the putative hPL pseudogene (hPL-1 or hPL-like gene). For the above purpose, we designed a strategy called homologous DNA mutagenesis, whereby we inserted the DNA regions of interest from the homologous genes into the context of the gene coding for hGH. A segment comprising the first 588 base pairs (bp) of the gene from the capping site to the second intron of the hGH gene was replaced by the corresponding segments from hPL-3 and hPL-4 genes. These 588-bp segments from the two active hPL genes display only eight nucleotidic changes distributed as follows: one in the 5'-untranslated region, one in the first exon (signal peptide), three in the first intron and three in the

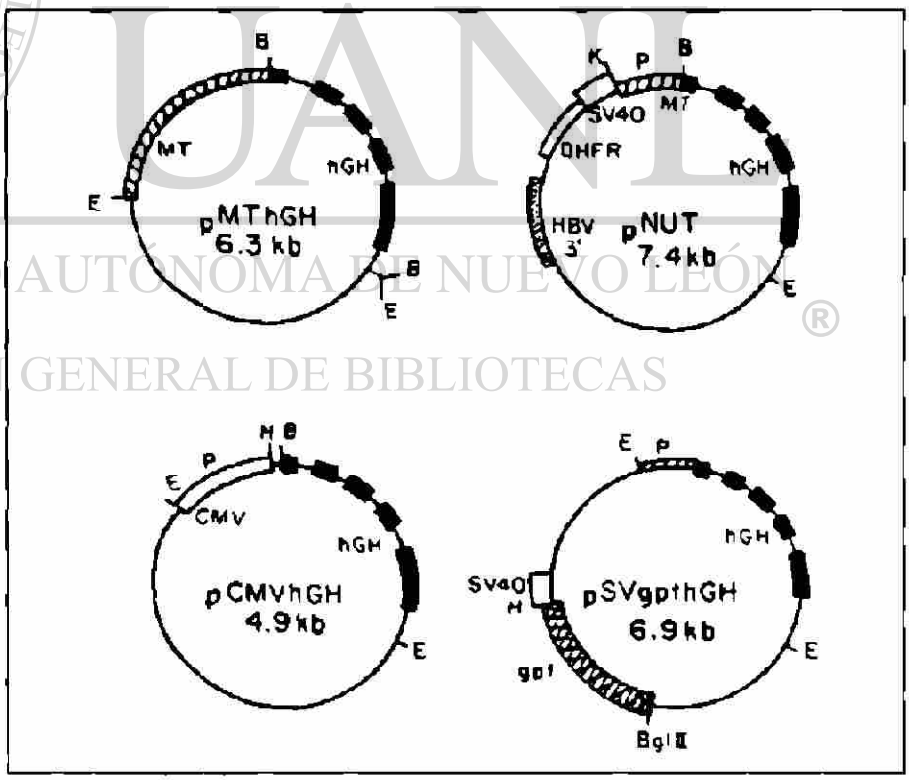

Figure 1. Structure of hGH expression vectors. With the exception of pSVgpthGH, which contains the entire hGH gene including its natural promoter, all other vectors shown here were constructed by fusing the hGH gene structural region between the *BamHI* site at the beginning of the first exon to the *EcoRI* site at the 3' end to different transcriptional control elements. In pMThGH, the expression of the hGH promoter-less gene is directed by the mouse metallothionein promoter. In pNUT, the SV40 enhancer and the mouse metallothionein promoter control the level of transcription. Finally, in pCMVhGH the transcriptional control element is the enhancer-promoter of the immediate early transcribed region of human cytomegalovirus

second intron. We also replaced this same region for sequences from the hPL-3m hybrid gene. hPL-3m ("m" means mutated) is a derivative from the active hPL-3 gene constructed in our laboratory (R. Ramírez-Solís and H.A. Barrera-Saldaña, unpublished results) carrying the point mutation at the splice site described above. Maps of these chimeric genes are shown in Figure 2.

Transient Expression Levels of the hGH Structural Genes Containing Mutations

To quantify the effects of the expression of the sequence changes introduced into the hGH structural gene after their construction, we inserted the hybrid genes into pNUT. Both the plasmids carrying the hybrid genes and control plasmids were introduced into COS-7 cultured cells. We found that the media from cells transfected with the plasmid carrying the hGH-N/hPL-3 hybrid gene [GH(PL-3: I, II)] contained hGH equivalent to 62.3% of that found with the positive control (pNUT). Values of hGH, five times lower than those obtained with the hGH-N/hPL-3 hybrid gene, were detected in the media of cells transfected with the hGH-N/hPL-4 hybrid gene [GH(PL-4: I, II)] plasmid. The hGH level in the media of cells transfected with the plasmid vector having as insert the hGH-N/hPL-3m hybrid gene [GH(PL-3m: I, II)] was negligible. These results (see Table 1) suggest that the splice site mutation at the beginning of intron II of the hPL-1 gene may be the cause of a reduced expression of this gene. In addition, they help to reveal sequence variations within the first 600 bp of the 2 active hPL genes as a possible source of difference in the hPL gene expression level.

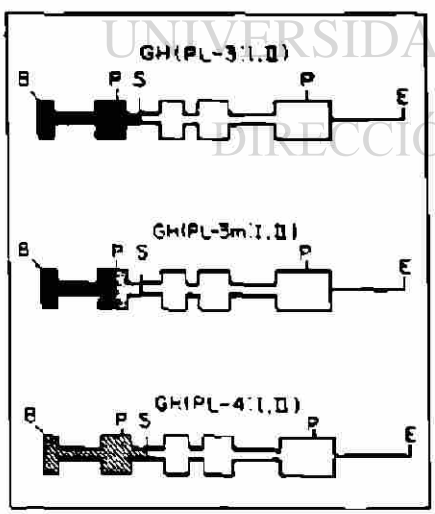

Figure 2. Genetic organization of chimeric gene constructs. *The drawings illustrate the* maps and origin of the gene fragments (2150 bp) used for the construction of the chimeric genes used. Boxes represent exons. Sites for restriction enzymes are: B = *Bam*HI, P = *Pvu*II, S = *Sac*I and E = *Eco*RI. Origins of gene fragments are in black, hPL-3; stippled, hPL-1; hatched, hPL-4; and open, hGH-N.

CONCLUSIONS

A novel aspect of our strategy is the use of hGH, a convenient extracellular reference protein (13) to quantify the effect of mutations on gene expression. It is particularly helpful when studying the expression of a gene related to the reporter gene. This is achieved by homologous DNA mutagenesis, which consists of the insertion of mutations present in members of a multigene family into the context of a homologous reporter gene. Using the gene coding for the well-known form of hGH, our approach permits a quantitative measurement of the effect of practically any mutation or nucleotide sequence change occurring in placental lactogen and growth hormone genes. Our procedure should be possible to use with any gene whose protein product can be easily detected.

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