ABRIR RESULTADOS

DISCUSIÓN

1. IMPLICACIÓN DE LA CD-E EN LA METÁSTASIS. RELACIÓN CON LA MMP-9.

1.1. CD-E y propiedades tumorales: Papel en la metástasis.

Es un hecho demostrado que la molécula de adhesión celular CD-E está implicada en el proceso tumoral en el sentido en que previene un comportamiento invasivo de las células tumorales, y que su ausencia induce un aumento en la invasividad celular (Behrens *et al.*, 1989; Frixen *et al.*, 1991; Vleminckx *et al.*, 1991). Este hecho está corroborado por diversos estudios sobre la expresión de CD-E en muestras clínicas de carcinomas de distinto origen y en líneas celulares derivadas de tumores donde la ausencia de CD-E se correlaciona con un menor grado de diferenciación y una mayor capacidad invasiva (Frixen *et al.*, 1991; Bussemakers *et al.*, 1992; Gamallo *et al.*, 1993; Takeichi, 1993; Umbas *et al.*, 1994; Oka *et al.*, 1996). En este sentido, los resultados obtenidos en este trabajo están en concordancia con la mayoría de estudios sobre el tema: la falta de CD-E (inducida mediante el bloqueo de su expresión por transfección con un cDNA antisentido de CD-E) induce en la línea celular E24 una mayor capacidad invasiva *in vitro* (Fig. 13).

La relación de la CD-E con el comportamiento metastásico, en cambio, no está tan bien establecida. Algunos estudios sobre expresión de CD-E en líneas celulares tumorales indican que la presencia o ausencia de CD-E es independiente de la capacidad metastásica (Bussemakers *et al.*, 1992; Caulín *et al.*, 1996). Por otro lado, numerosos estudios clínicos indican que aquellos tumores con capacidad de metastatizar muestran menor expresión de CD-E (Schipper *et al.*, 1991; Umbas *et al.*, 1992; Mayer *et al.*, 1993; Oka *et al.*, 1993; Katagiri *et al.*, 1995;), mientras que también existen estudios clínicos en los que no se observa dicha correlación (Oka *et al.*, 1992; Gamallo *et al.*, 1996; Zschiesche *et al.*, 1997). Sin embargo, en nuestro conocimiento, tan solo existía un trabajo en el que se relacionaba de forma directa la ausencia de CD-E con el comportamiento metastásico en líneas de cáncer de mama (Mbalaviele *et al.*, 1996). En este trabajo se muestra cómo la transfección de CD-E en la línea celular de cáncer de mama MDA-MB-231, que provoca metástasis en hueso, disminuye de forma considerable su agresividad y su capacidad metastásica.

Nuestros resultados muestran claramente que la pérdida de CD-E en la línea celular E24, tumorogénica pero no metastásica, es capaz de provocar en la misma un comportamiento metastásico (Tabla IV). En este sentido, es uno de los primeros trabajos, junto con el del grupo de Mbalavile, en donde se muestra una correlación directa y causal entre la presencia o ausencia de CD-E y la capacidad metastásica.

Debido a la naturaleza multicausal del cáncer y de la metástasis, esta correlación no tiene porqué suceder siempre, ni en todos los tipos de carcinomas. El hecho tumoral y metastásico depende de numerosos factores y de la combinación de los mismos, por lo que uno sólo aisladamente (como puede ser la presencia o ausencia de CD-E) no es suficiente para poder predecir el comportamiento metastásico de un determinado tipo de tumor o línea

celular. En cualquier caso, la presencia o ausencia de CD-E, sí puede ser capaz, en algunas circunstancias, de desviar la balanza en un sentido o en otro.

Así, ni siquiera en el sistema de la carcinogénesis de piel de ratón la presencia de CD-E es siempre suficiente para evitar un comportamiento metastásico. De hecho, la línea celular E62 expresa CD-E y es, en cambio, metastásica (Caulín *et al.*, 1996). El comportamiento metastásico de esta línea celular podría explicarse por los niveles elevados de v-Ha-ras que presenta, a diferencia de E24 donde los niveles de v-Ha-ras son mucho menores (Caulín *et al.*, 1996). De hecho, se ha demostrado que células transformadas con ras se vuelven tumorogénicas y en algunos casos metastásicas (Mareel *et al.*, 1991) y que los niveles de Ha-ras corresponden muchas veces con el comportamiento maligno (Brissette *et al.*, 1993; Chambers y Tuck, 1993).

Otra posible explicación para el hecho de que E62 sea metastásica es la posibilidad de que exista una "downregulación" transitoria de la función o expresión de CD-E durante el proceso metastásico, que permita a las células liberarse del tumor primario y poder migrar por el torrente circulatorio. De hecho, el fenómeno de la inestabilidad de la expresión de CD-E *in vivo* se ha evidenciado ya en otros sistemas. Así, Mareel *et al.* (1991) obtuvieron evidencias de que factores del huésped *in vivo* pueden inducir pérdida de CD-E: la línea célular MDCK-ras-e (MDCK transformada con ras) que en cultivo expresaba CD-E de forma homogénea y no era invasiva, cuando se inyectaba en ratones, se volvía invasiva y metastásica, y tanto los tumores primarios como las metástasis eran heterogéneos, con zonas donde se expresaba CD-E y zonas CD-E negativas. Cuando se cultivaban in vitro explantes de estos tumores, después de varios pases las células expresaban CD-E homogéneamente. Estos experimentos sugieren que en el huésped existen factores que pueden "downregular" la expresión de CD-E. Esta heterogeneidad en la expresión en CD-E en relación con el comportamiento invasivo y metastásico no es un hecho aislado y ocurre asimismo en líneas de carcinoma de ovario en cultivo (Hashimoto *et al.*, 1989) y en carcinomas de diverso origen (Shimoyama y Hirohashi, 1991a y 1991b; Bussemakers *et al.*, 1992). Otro posible mecanismo es una disminución de la función de CD-E sin pérdida de expresión, por ejemplo por mecanismos moleculares como fosforilación o defosforilación de las cateninas, que pueden modular las interacciones celulares (Kemler, 1993), o también por modulaciones en los niveles de β-catenina o plakoglobina, que pueden afectar a las rutas de señalización mediada por ellas. Estos fenómenos podrían jugar un papel clave en la invasión y metástasis.

Si este es el caso, es lógico que al eliminar la CD-E, como ocurre en los transfectantes antisentido de CD-E, las células puedan metastatizar mucho más fácilmente, puesto que se favorecen las condiciones para escapar del tumor primario. Si a este hecho se une además que con la pérdida de CD-E las células adquieren una mayor capacidad degradativa de la matriz extracelular (como se discutirá más adelante), las posibilidades de metastatizar son aún mayores.

Para nosotros era importante, a la hora de otorgar a la ausencia de CD-E el papel causal en la metástasis, analizar los niveles de v-Ha-ras, puesto que es posible que hubiera una heterogeneidad poblacional en las células E24, y que por el proceso de la transfección y selección con puromicina se hubieran seleccionado aquellas células con altas dosis de v-Ha-ras. En este caso, no podríamos diferenciar entre los efectos producidos por altas dosis de v-Ha-ras y los producidos por la ausencia de CD-E. Los análisis de Northern-blot (Tabla V) demostraron que los niveles de v-Ha-ras no se habían alterado tras la transfección, y los transfectantes mantenían niveles bajos de v-Ha-ras, similares a los mostrados por la línea parental E24.

Por otro lado, tampoco creemos que los efectos observados puedan deberse a un efecto inespecífico de bloqueo de la expresión de cualquier otro gen por el cDNA antisentido, ya que la molécula de mayor homología presente en estas líneas de queratinocitos, la CD-P, no veía alterada su expresión (Fig. 11 y Fig. 12B).

1.2. Mecanismos implicados en la mayor capacidad metastásica e invasiva por la pérdida de CD-E: Papel de MMP9.

Puesto que los transfectantes antisentido eran más invasivos y metastásicos, nos preguntamos si la simple pérdida de adhesión intercelular era suficiente para provocar el comportamiento más agresivo de las células o si, por el contrario, la pérdida de CD-E provocaba en las mismas algún cambio adicional que contribuyera a la malignidad. Como se indicó en la Introducción, en el fenómeno metastásico juegan un papel esencial los procesos de degradación de la matriz extracelular, en los que intervienen principalmente proteasas del grupo de las metaloproteinasas de matriz (MMPs) y el activador de plasminógeno tipo urokinasa (uPA). Por ello, decidimos investigar la actividad de estas proteasas en nuestro sistema celular.

Un estudio previo de Frixen y Nagamine (1993) había puesto de manifiesto que el tratamiento de células de carcinoma de mama con DECMA (un anticuerpo bloqueante de la función de CD-E) inducía un aumento en los niveles de uPA secretado al medio extracelular, que era el responsable de la mayor capacidad invasiva que presentaban las células tras el tratamiento con DECMA. En este sentido nos pareció lógico pensar que un mecanismo similar funcionara también en las células E24 transfectadas con el vector antisentido de CD-E, y que un bloqueo de la función de CD-E indujera aumento en la secreción de uPA. Sin embargo, la medida de actividad uPA en el medio condicionado y en el extracto celular indicó que el uPA no parecía ser el responsable del aumento de invasividad en los transfectantes antisentido de CD-E (Fig. 14). Las diferencias entre ambos estudios pueden deberse a los diferentes sistemas experimentales: distintas proteasas pueden ser responsables de la invasividad en tumores de distinto origen.

Por el contrario, el estudio de la actividad gelatinolítica de las líneas celulares de queratinocitos sí reveló una interesante relación entre la presencia de CD-E y la actividad

gelatinolítica MMP-9: aquellas líneas que expresaban CD-E apenas mostraban actividad MMP-9 y las que no expresaban CD-E poseían una alta capacidad degradativa por MMP-9 (Fig. 15A y Fig. 16A). Por los sistemas experimentales empleados: transfección de CD-E en una línea que no la expresa (HaCa4) y transfección de un cDNA antisentido de CD-E en una línea que sí la expresa (E24) y análisis de los clones generados, ésto no parece una simple correlación, sino una relación causal. Queda por determinar con exactitud si la regulación ejercida por CD-E sobre la expresión de MMP-9 es a nivel transcripcional o post-transcripcional, ya que, mientras en los clones originados por la transfección de HaCa4 la expresión de CD-E induce una disminución notable en los niveles de mRNA de MMP-9 (Fig. 16B), el bloqueo de la expresión de CD-E en E24 (clones antisentido) sólo es capaz en algunos casos (clon P1-5) de inducir un incremento considerable en los niveles de mensajero de MMP-9 (Fig. 15B). Deben existir, por tanto, mecanismos postranscripcionales (por ej., estabilidad del mensajero o de la proteína, control de traducción o control de secreción) responsables del aumento de actividad MMP-9 en los clones P1-10 y P1-13. Esto sugiere que el control que ejerce CD-E sobre MMP-9 se realiza a varios niveles, aunque el mecanismo exacto lo desconocemos actualmente, y debe ser objeto de posteriores estudios. En cualquier caso, parece que el aumento de la actividad MMP-9 en los transfectantes antisentido se debe a un efecto específico originado por la pérdida de adhesión mediada por CD-E, y no a la simple pérdida de contactos celulares, ya que estas células siguen manteniendo contactos entre sí, por la presencia de CD-P.

Nuestros resultados comparten cierta analogía con los obtenidos por el grupo de Takeichi (Miyaki *et al.*, 1995), que transfectó el cDNA de CD-E en una línea celular de carcinoma de colon altamente invasiva. Algunas de las líneas generadas, además de presentar menor capacidad invasiva mostraron un descenso significativo en la secreción de una gelatinasa de 62 KDa, correspondiente con la MMP-2. Estos estudios, junto con los resultados mostrados en esta Tesis, están de acuerdo con una regulación de MMPs por parte de la CD-E. La diferencia en el tipo de MMP está de acuerdo con la idea de que diferentes proteasas juegan un papel prioritario en diferentes tipos tumorales. De hecho, la línea parental empleada en el trabajo de Miyaki no mostraba actividad gelatinolítica detectable MMP-9. En nuestras líneas celulares de queratinocitos de ratón, el examen de otras MMPs mostró que la principal gelatinasa expresada era MMP-9, mientras que los niveles de MMP-2, MMP-3 y MMP-7 eran prácticamente indetectables, por lo que no creemos que jueguen un papel esencial en este sistema de carcinogénesis química de piel de ratón.

Por otro lado, ya se había sugerido, en otros sistemas experimentales, que la presencia de CD-E podía alterar la expresión de algunos genes, como es el caso de la inducción de la expresión de proteínas desmosomales en una línea celular de epitelio de retina tras la transfección con CD-E (Marrs *et al.*, 1995). Recientemente Larue *et al.* (1996) emplearon un sistema experimental, basado en la manipulación de células embrionarias stem (ES), para demostrar que distintos tipos de cadherinas podían dirigir la formación de

distintas estructuras tisulares (epitelio por CD-E). Además observaron que la CD-E inducía represión en la expresión del factor de transcripción mesenquimal T-brachiury, sugiriendo que la presencia o ausencia de CD-E influye en una actividad génica específica.

Teniendo en cuenta estos datos, junto con los obtenidos en esta Tesis, es tentador especular sobre un posible mecanismo por el que la CD-E participaría en la transmisión de una señal que en último término tendría una consecuencia transcripcional, para poner en marcha un determinado programa. Las recientes evidencias sobre la participación de la β-catenina en señalización celular (Gumbiner *et al.*, 1995) permiten hipotetizar que la CD-E actúa controlando los niveles de β-catenina o plakoglobina libre que es encargada de transmitir la señal al núcleo. En este sentido, sería de gran utilidad examinar en los transfectantes antisentido de CD-E la distribución de β-catenina y plakoglobina: nuclear, citosólica o anclada a complejos de CD-P en contactos celulares.

2. MECANISMOS QUE CONTROLAN LA EXPRESIÓN DE CD-E EN LA CARCINOGÉNESIS DE PIEL DE RATÓN.

El hecho de que en muchos carcinomas indiferenciados se pierda la expresión de CD-E, y el posible papel causal que ésta tiene en la malignidad, hace que sea de gran interés el estudio de los mecanismos que controlan la expresión de esta molécula. Además, el conocimiento de tales mecanismos puede servir para comprender mejor las bases de la expresión específica de tejido epitelial, ya que la CD-E se expresa exclusivamente en tejidos epiteliales en el organismo adulto.

Estudios previos sobre el promotor de CD-E, empleando principalmente líneas de carcinoma de mama y de glándula mamaria normal (Behrens *et al.*, 1991) habían mostrado que un fragmento del gen de CD-E de ratón que comprendía las posiciones -178/+92, respecto del sitio de inicio de la transcripción, mostraba actividad transcripcional específica. La especificidad epitelial vendría dada por el elemento palindrómico E-pal, que se proponía que estimulaba la transcripción en líneas epiteliales y la inhibía en líneas que no expresan CD-E. Sin embargo, no se observaron diferencias en los factores nucleares expresados por líneas CD-E positivas y negativas por experimentos de unión a DNA *in vitro* (footprinting *in vitro* y retardo en gel) (Behrens *et al.*, 1991). Resultados similares se observaron con una región homóloga del gen de CD-E humana (Bussemakers *et al.*, 1994a).

Nosotros pensamos que el modelo de la carcinogénesis de piel de ratón podía ser un sistema útil para profundizar en el conocimiento de los mecanismos de control de la expresión de CD-E, ya que proporciona una serie de líneas celulares que representan distintos estadíos de la progresión tumoral y donde la expresión de cadherinas se asemeja a lo que sucede *in vivo* durante la carcinogénesis de piel. Además, en concreto, el conocimiento de los eventos transcripcionales que llevan a la línea celular E24 a reexpresar CD-E podía ser de gran utilidad para comprender los mecanismos que inducen las

características peculiares de menor agresividad que muestra esta línea celular respecto a la línea parental HaCa4.

Hay dos mecanismos principales que, a grandes rasgos, pueden hacer que un gen se transcriba o se mantenga reprimido, que no tienen porqué ser excluyentes entre sí, y que, de hecho, puede afectarse mutuamente: a) la presencia de una serie de factores trans (factores de transcripción o cofactores) que pueden influir estimulando o reprimiendo la transcripción, generalmente por interacción con la maquinaria basal de transcripción; y, b) la estructura misma del DNA y la cromatina en la región que rodea al gen (factores que actúan en cis). En este último caso, mecanismos como el empaquetamiento de la cromatina en nucleosomas o la metilación endógena del DNA en islas CpG pueden reprimir la actividad transcripcional de un gen (Cedar, 1988; Counts y Goodman, 1995).

En el estudio de la regulación transcripcional del gen de CD-E de ratón abordado en la presente memoria nos hemos centrado principalmente en los mecanismos que actúan en trans, y en concreto a través de la región comprendida entre las posiciones -178/+92 del promotor de CD-E, que había mostrado actividad específica en otros sistemas celulares (Behrens *et al.*, 1991).

2.1. Región -178/+92. Elementos reguladores.

Los estudios de transfección transitoria en los queratinocitos de ratón mostraron que la región -178/+92 del promotor de CD-E posee actividad transcripcional específica celular: es activa en líneas que expresan CD-E e inactiva en líneas de fenotipo fibroblastoide que no expresan CD-E (Fig. 18). La línea epiteloide HaCa4, CD-E negativa, mantiene un cierto nivel, aunque reducido, de actividad promotora. Este dato quizás puede tener relación con el hecho de que, bajo ciertas circunstancias, esta línea celular puede reactivar la expresión de CD-E, como ocurre esporádicamente en células aisladas en cultivo a confluencia o tras su inyección en ratones desnudos (Navarro *et al.*, 1991), indicando que la pérdida de CD-E no es un hecho irreversible en esta línea celular.

Asimismo, hemos encontrado en esta región elementos reguladores positivos: región rica en GC y caja CCAAT, así como secuencias reguladoras negativas, como el elemento E-pal, que actúa en todas las líneas de queratinocitos examinadas. Estas observaciones están de acuerdo con otros estudios sobre el promotor de CD-E en otros sistemas, si bien parece que el elemento E-pal en células de carcinoma de mama que expresan CD-E juega un moderado papel regulador positivo (Behrens *et al.*, 1991; Hennig *et al.*, 1996). Además, hemos encontrado otra región reguladora, que a través de un sitio de unión a factores de la familia Ets, actúa como modulador negativo de la transcripción en células de queratinocitos que expresan CD-E.

2.1.1. Elemento represor E-pal.

El efecto represor del elemento E-pal es mayor en las líneas que no expresan CD-E y es particularmente intenso en la línea fusiforme CarB (Fig. 19), representante del último estadío de la progresión tumoral. Por otra parte, este elemento se encuentra protegido en experimentos de footprinting *in vitro* con extractos celulares de todas las líneas analizadas, pero de forma diferente en líneas que expresan y que no expresan CD-E (Fig. 21). Los experimentos de retardo en gel, sin embargo, muestran que en todas las líneas existe un factor/es que forma un complejo similar que puede unirse a este elemento. Estas aparentes discrepancias pueden explicarse por la existencia de factores similares pero con distintas propiedades transcripcionales en líneas que expresan y no expresan CD-E, por modificaciones diferenciales en el factor o por la interacción con diferentes cofactores. En cualquier caso, los experimentos de footprinting *in vivo* (Fig. 34 y 35) indican que en las líneas que no expresan CD-E, una de las pocas posiciones protegidas es la posición central del elemento E-pal, lo que apoya la idea de un predominante papel represor del elemento E-pal en células CD-E negativas.

La existencia de dos cajas E en el elemento E-pal sugieren que el potencial elemento represor que se une a este elemento podría pertenecer al grupo de factores de transcripción bHLH, que se unen a cajas E (secuencia consenso CANNTG). De esta forma, diferencias en los tipos de factores, en los heterodímeros formados o en posibles cofactores o proteínas de interacción darían cuenta del efecto diferencial entre líneas positivas y negativas. Alternativamente, el posible factor de transcripción bHLH podría ser desplazado de su interacción con el elemento E-pal, en mayor o menor medida por un inhibidor, ejerciendo así el efecto represor. Los niveles y propiedades de este factor inhibidor serían los responsables en este caso del efecto diferencial. Otra posible opción es que el factor/es que se unen al elemento E-pal no esté relacionado con los factores de transcripción del tipo bHLH. Con los datos que disponemos actualmente no podemos discernir cual de las posibilidades es la correcta, aunque sí podemos hipotetizar sobre posibles mecanismos.

La hipótesis de un factor tipo bHLH cobra más fuerza si tenemos en cuenta los efectos que la proteína de adenovirus E1a produce en algunas líneas celulares. E1a es capaz de inducir una conversión hacia un fenotipo epiteloide en una variedad de líneas fibroblastoides de diverso origen, y en concreto, induce la expresión de CD-E en una línea celular de rabdomiosarcoma que no la expresaba (Frisch, 1994). En nuestro laboratorio también hemos observado que E1a puede inducir la expresión de CD-E en la línea celular MSC11A5 (Sánchez-Prieto *et al.*, 1996 y resultados no mostrados). Por otra parte, se ha descrito que E1a es capaz de interaccionar con el dominio bHLH de algunos factores de este tipo, como miogenina y E12, reprimiendo así la expresión promovida por estos factores e inhibiendo la diferenciación miogénica (Taylor *et al.*, 1993). Por tanto, es tentador especular que el mecanismo por el que E1a induce la expresión de CD-E en algunos sistemas es

mediante la liberación del bloqueo en su expresión ejercido a través del elemento E-pal por factores del tipo bHLH.

Por otro lado, la caja E situada en la mitad 5' del elemento E-pal contiene la secuencia CACCT. Se ha descrito que el represor δEF1 se une específicamente a estas secuencias contenidas en algunas cajas E, compitiendo con los factores activadores bHLH por la unión a la caja E y ejerciendo así un efecto represor (Sekido *et al.*, 1994). El represor δEF1 se expresa durante el desarrollo embrionario principalmente en derivados mesodérmicos (miotomo) y tejido neural (notocorda) (Sekido *et al.*, 1994). La idea de un elemento represor que compite con los factores activadores bHLH podría explicar el efecto regulador positivo del elemento E-pal observado por el grupo de Birchmeier en líneas que expresan CD-E (Behrens *et al.*, 1991; Hennig *et al.*, 1996). Sin embargo, en nuestro sistema experimental se observa un incremento en la actividad tras la mutación del elemento E-pal, también en células CD-E positivas (Fig. 19). Estos datos sugieren más bien que el inhibidor tiene un efecto represor *per se* y no por desplazamiento de un activador que actúe a través del elemento E-pal.

La compartimentalización de los factores de la familia Myc/Max en las distintas capas de la epidermis (Hurlin *et al.*, 1995a; Hurlin *et al.*, 1995b; Gandarillas y Watt, 1995) junto con su papel en procesos de diferenciación, proliferación y apoptosis (Amati y Land, 1994) nos hizo pensar que estos factores, de la familia bHLH, pudieran intervenir en la represión mediada por E-pal. Además, la posibilidad de formar heterodímeros con diferentes miembros, que activan o reprimen la transcripción podría ayudar a crear el efecto diferencial en células CD-E positivas y CD-E negativas. Sin embargo nuestros resultados preliminares mediante estudios de cotransfección con miembros de esta familia (Tabla VIII) no nos permiten confirmar esta hipótesis.

Por otra parte, en el elemento E-pal también existen sitios consenso de unión de los factores tipo dedos de zinc Snail (Mauhin *et al.*, 1993) y Escargot (Fuse *et al.*, 1994) descritos por primera vez en *Drosophila*, pero con funciones también en organismos superiores. Los factores Snail y Slug son muy similares y tienen un patrón de expresión en desarrollo muy peculiar, en zonas de migración de la cresta neural y en zonas de invaginación del ectodermo para formar derivados mesodérmicos (Nieto *et al.*, 1994). Estos datos nos inclinaron a pensar que factores del tipo Snail/Slug podrían participar en la disminución de la expresión de la CD-E durante la progresión tumoral, que en cierto modo es una transición epitelio-mesénquima. Los resultados obtenidos mediante cotransfección de estos factores indicaron que quizás en la línea celular HaCa4 factores de este tipo pueden jugar un papel en el bloqueo de la expresión de CD-E, pero no en líneas de queratinocitos que expresan CD-E, como la línea celular MCA3D (Tabla VIII). De hecho, la transfección estable de Snail, y en mayor medida de Slug, induce en la línea celular HaCa4 un cambio hacia un fenotipo fibroblastoide, con pérdida de contactos y emisión de prolongaciones citoplásmicas (resultados no mostrados), aunque no hemos estudiado con más detalle los

cambios producidos. Estas observaciones estarían de acuerdo con resultados recientes en células de vejiga de rata, NBT-II, que muestran que Slug puede jugar un papel en las transiciones epitelio-mesénquima (Savagner *et al.*, 1997). Sin embargo, en las líneas MCA3D y PDV la transfección de Slug de forma estable provoca un incremento de los niveles de CD-E y de CD-P, detectados por RT-PCR (resultados no mostrados, en colaboración con A. Nieto, Instituto Cajal), hecho que está de acuerdo con los resultados obtenidos en los experimentos de transfección transitoria en la línea MCA3D (Tabla VII). En concordancia con el papel positivo que pueden tener factores de este tipo sobre la regulación de CD-E en algunos tipos celulares, por ejemplo MCA3D, recientemente se ha demostrado que el factor Escargot controla de forma positiva la expresión de CD-E en *Drosophila* (Tanaka-Matakatsu *et al.*, 1996). La secuencia de unión a Escargot (G/ACAGGTG) contiene una caja E y en células de *Drosophila* Escargot bloquea la activación transcripcional inducida por un heterodímero de proteínas bHLH (Fuse *et al.*, 1994), sugiriendo que Escargot puede regular procesos dependientes de proteínas bHLH.

Todos estos datos dan una idea de la compleja naturaleza de las interacciones entre factores de transcripción y sus inhibidores. La diferencia de comportamiento entre diferentes líneas celulares puede deberse a la diferente combinación de factores endógenos que estaría en relación con el estadío de diferenciación en que se encuentren, y podría promover la conversión hacia un fenotipo mesenquimal o epitelial en cada caso.

2.1.2. La región GC y la caja CCAAT regulan positivamente la transcripción.

Los ensayos de transfección transitoria del promotor de CD-E (Fig. 18 y Fig. 20) indicaron que tanto la región rica en GC como la caja CCAAT del promotor de CD-E actúan como reguladores positivos de la transcripción de CD-E en las líneas analizadas. Aunque todas líneas de queratinocitos poseían factores capaces de unirse a estas regiones, tal como indican los ensayos de retardo en gel con las sondas CCAAT-E y GC-E (Fig. 24 y Fig. 25), sin embargo deben existir diferencias en la naturaleza de los factores o cofactores que interaccionan con estas regiones entre líneas CD-E positivas y CD-E negativas, como lo evidencia el menor patrón de protección observado en los experimentos de footprinting *in vitro* con extractos de líneas CD-E negativas (Fig. 21). Además, *in vivo*, estas regiones se encuentran ocupadas por factores sólamente en aquellas líneas que expresan CD-E, tal como lo evidencian los ensayos de footprinting *in vivo* (Fig. 34 y 35), lo que sugiere que estas regiones sólo ejercen su efecto estimulador en estas líneas.

Los resultados de super-retardo en gel (Fig. 26) muestran con claridad que los factores de transcripción SP1 y AP2 son capaces de unirse a la región rica en GC de CD-E, y por tanto es muy probable que jueguen un papel importante en la regulación de la transcripción de CD-E.

La aparente discrepancia en los resultados sobre unión de factores en los ensayos de retardo en gel y de footprinting *in vitro*, pueden explicarse por la existencia en unas líneas y otras de diferentes cofactores o factores de transcripción que modulen su interacción con el DNA, y posiblemente también su actividad funcional, y que sólo en el contexto de una región amplia de DNA pueda evidenciarse su efecto, originando un patrón de protección diferente.

En cualquier caso, los factores de unión no parecen ser idénticos, como lo evidencia el diferente patrón de competición de los complejos generados por la región GC con extractos nucleares de PDV (CD-E positiva) y de CarB y HaCa4 (CD-E negativas) (ver Fig. 25). En este sentido, se ha descrito la existencia de diferentes isoformas del factor AP2, reguladas durante el desarrollo (Meier *et al.*, 1995; Chazaud *et al.*, 1996; Moser *et al.*, 1997). De igual manera también se han encontrado diversas isoformas de SP1 durante el desarrollo embrionario de ratón (Saffer *et al.*, 1991), que sugieren que SP1 podría tener un papel en procesos de diferenciación, además de su papel general en la transcripción de genes "housekeeping".

Chen *et al.* (1997) proponen que la relación entre las concentraciones de SP1 y AP2 puede modular la transcripción de la citoqueratina K3, que se expresa en las capas diferenciadas del epitelio de la cornea. El promotor de la K3 contiene sitios solapantes SP1 y AP2, donde SP1 activa y AP2 reprime la transcripción. Cuando se inducen a diferenciar células basales epiteliales indiferenciadas de córnea de conejo la relación de actividades SP1/AP2 de unión a DNA aumenta, coincidiendo con la activación del gen de K3 y la represión del gen de K14, citoqueratinas de capas diferenciadas y basales del epitelio de córnea, respectivamente. Un mecanismo similar podría tener lugar en la epidermis. En este sentido, el análisis por inmunoblot de los niveles de SP1 en las distintas líneas de queratinocitos no reveló diferencias en las cantidades de SP1, apareciendo en todos los casos dos bandas, resultantes probablemente de diferente grado de fosforilación (Jackson *et al.*, 1990) (resultados no mostrados). Los intentos de detección del factor AP2 con el anticuerpo de que disponíamos fueron fallidos. La idea de que AP2 pueda modular negativamente la transcripción, compitiendo con SP1 por la unión al DNA, no es contradictoria con nuestros resultados. Así, las mutaciones de las construcciones mut GC1 y mut GC2 (que son menos activas que la construcción silvestre, Fig. 20) pueden afectar a la unión a SP1, y no exclusivamente a la unión a AP2. Por otro lado, y por el hecho de que los sitios de unión a SP1 y AP2 de la región rica en GC son prácticamente solapantes (ver Fig. 27), el efecto inhibidor de la cotransfección con el dominante negativo de AP2 (Tabla IX), podría deberse también al desplazamiento de factores SP1, y no sólo al desplazamiento de AP2. En apoyo de esta hipótesis está el hecho de que el factor AP2 transmite los efectos de promotores tumorales tipo ésteres de forbol (Imagawa *et al.*, 1987), y esto podría representar uno de los mecanismos de represión de la CD-E en la carcinogénesis.

Otro posible mecanismo de regulación de la actividad transcripcional de CD-E a través de la región GC, es la posible interacción entre SP1 y AP2 con miembros de la familia Ets. Este mecanismo se disctutirá con más detalle en el siguiente Apartado.

2.1.3. Papel regulador negativo del sitio Ets.

Tanto los experimentos de footprinting *in vitro* y retardo en gel (Fig. 21 y 28) como los experimentos de footprinting *in vivo* (Fig. 34 y 35) sugieren la existencia de factores que se unen al promotor de CD-E, en la que hemos denominado región ECE, de forma diferencial en células que expresan y no expresan CD-E y que actuarían predominantemente en células CD-E positivas. La identificación del factor/factores que interaccionan con esta región como miembros de la familia Ets viene dada porque la mutación en el sitio EtsA que existe en esta región impide la unión de los factores al DNA (Fig. 29), y además tiene un efecto transcripcional en células CD-E positivas, induciendo un aumento en la actividad promotora (Fig. 30). Este último dato permite suponer que el papel del elemento Ets en estas líneas celulares es como modulador negativo de la transcripción.

El diferente patrón que muestran células CD-E positivas y negativas en experimentos de footprinting *in vitro* y de retardo en gel, sugiere que este elemento pueda tener alguna implicación en la expresión específica celular, y posiblemente también en la expresión específica epitelial. De hecho, se han caracterizado muy recientemente miembros de esta familia exclusivos de células epiteliales, como los factores ESE-1 y jen (Oettgen *et al.*, 1997; Andreoli *et al.*,1997).

Se sabe que la mayoría de los factores tipo Ets establecen interacciones con otros factores de transcripción, en muchos casos con AP1, para ejercer su efecto transcripcional (ver Introducción , Aptdo. 6.3). Recientemente también se han encontrado promotores en los que Ets actúa interaccionando con el factor de transcripción SP1 (Gégonne *et al.*, 1993; Dittmer *et al.*, 1994; Lee *et al.*, 1996; Ansieau *et al.*, 1997). Los experimentos de competición de la unión al oligonucleótido ECE mostrados en la Fig. 28C, junto con resultados no mostrados nos inclinan a pensar que en el promotor de CD-E factores tipo Ets están también actuando mediante interacción con otros factores de transcripción, posiblemente SP1 o AP2. Así, el complejo de retardo mayoritario formado con el oligonucleótido ECE se competía específicamente con los oligonucleótidos consenso para SP1 (SP1-SV40) y para AP2 (AP2-SV40) y también en parte con el oligonucleótido consenso de unión al factor CP1, pero no con otros oligonucleótidos probados irrelevantes o de unión a otros factores de transcripción (resultados no mostrados). Estos resultados, junto con los datos existentes en la literatura, sugieren que factores Ets están formando parte de grandes complejos transcripcionales, implicados en la regulación fina de la transcripción.

Por otro lado, se ha descrito que algunos factores de esta familia poseen un dominio inhibidor de la unión al DNA (Wasylyk *et al.*, 1992). Este hecho podría explicar la

competición por el oligonucleótido PEA 3 de la banda de mayor movilidad que aparece en la Fig. 28C, así como el hecho de que esta banda no se defina con claridad en todos los experimentos. Esta banda podría tratarse de un producto de degradación de Ets, quizás sin el dominio inhibidor, y tendría propiedades de unión al DNA diferentes respecto al factor completo que, además, estaría formando interacciones con otros factores nucleares. Por tanto, esta banda podría ser competida más fácilmente por un oligonucleótido consenso para factores Ets, aunque no por el oligonuclótido mutado.

Los intentos de identificación de factores Ets en experimentos de super-retardo, con un anticuerpo anti-Ets1, fueron fallidos. Sin embargo, se ha descrito que en otros sistemas celulares anticuerpos anti-Ets no interfieren siempre con la formación de complejos Ets transcripcionalmente activos (Lee *et al.*, 1996; Wu *et al.*, 1994).

Si el elemento Ets ejerce, como hemos indicado, un papel modulador negativo en líneas que expresan CD-E (Fig. 30), cabría esperar, por tanto, que la transfección de ERM indujera un descenso en la actividad transcripcional. Sin embargo, esto no ocurre así (Fig. 31). Una posible explicación para este hecho es que el factor ERM, exógeno, no es el inhibidor endógeno que está actuando, sino que tiene propiedades diferentes, y ERM está desplazando al inhibidor endógeno, liberando por tanto el efecto represor.

En este sentido, sería de gran utilidad conocer qué miembros de la familia Ets se expresan en los queratinocitos de ratón analizados. Los estudios de inmunoblot realizados (Fig. 32) no permiten distinguir entre los diferentes miembros de la familia y, además, el anticuerpo empleado no es capaz de detectar a todos los miembros de esta amplia familia. Por tanto, sería útil realizar ensayos de Northern-blot o de RT-PCR con sondas específicas para cada miembro de la familia. Es interesante destacar que Baert *et al.* (1997) han encontrado alta expresión de los miembros de la familia Ets, ERM y ER81 en líneas de carcinoma de mama que son CD-E negativas, mientras que otras líneas de carcinoma de mama que son CD-E positivas no muestran expresión de ninguno de estos dos factores. Si la presencia o ausencia de estos factores puede tener alguna consecuencia en la transcripción de CD-E aún está por determinar.

Por otra parte, los resultados mostrados en la figura 31 para la línea celular HaCa4, parecen indicar que factores de esta familia también pueden regular la actividad transcripcional en líneas CD-E negativas como HaCa4, pero a través de sitios diferentes al sitio EtsA. ERM también fué capaz de aumentar la actividad promotora de la construcción -58 en HaCa4 (resultados no mostrados). El análisis de secuencias contenidas en esta construcción indicó la presencia de otros sitios posibles de unión a Ets entre las posiciones +80 y +30, que unían proteínas nucleares en experimentos de retardo en gel (datos no mostrados) que quizás pudieran ser los responsables del efecto transcripcional ejercido por ERM en la línea HaCa4.

2.2. Promotor endógeno de CD-E. Mecanismos reguladores de su transcripción en queratinocitos de ratón.

El estudio de la metilación endógena del DNA en diferentes líneas celulares de queratinocitos de ratón indicó que el DNA está metilado en la región 5' del gen de CD-E en todas las líneas celulares analizadas (Fig. 33), independientemente de su nivel de expresión de CD-E. Estos resultados contrastan con los obtenidos por los grupos de Hirohashi y Baylin (Yoshiura *et al.*, 1995; Graff *et al.*, 1995) que observaron metilación endógena en la región 5' del gen de CD-E en líneas humanas de carcinomas desdiferenciados de distinto origen que no expresaban CD-E, pero no en líneas de carcinomas diferenciados, CD-E positivas, ni en tejido normal de mama. Además, estos autores observaron que algunas líneas eran capaces de expresar CD-E tras el tratamiento con el agente demetilante 5-aza-citidina, por lo que sugieren que la metilación endógena del DNA alrededor de la región promotora de CD-E es uno de los mecanismos principales que reprimen su expresión en la carcinogénesis. Asimismo, el grupo de Birchmeier también observó metilación endógena en algunas líneas humanas de carcinoma CD-E negativas y en fibroblastos de ratón NIH3T3, pero no en líneas humanas de carcinoma CD-E positivas ni en una línea epitelial de glándula salivar de ratón, CSG (Hennig *et al.*, 1995). Sin embargo, también se había indicado que líneas de carcinoma de mama desdiferenciadas CD-E negativas mostraban menor grado de metilación que las diferenciadas (Frixen *et al.*, 1991). Esta, en general, aparente discordancia con nuestros resultados puede deberse a los diferentes sistemas experimentales empleados: nuestros estudios se centran en la carcinogénesis de piel, mientras que ninguno de los otros grupos estudió queratinocitos o pieles normales o tumorales. Por otra parte, Ji *et al.* (1997) aporta datos sobre la regulación del gen de CD-E humano que sugieren que el estado endógeno de metilación no es tan esencial para la expresión o no de la CD-E: en sus manos el tratamiento con 5-aza-citidina de líneas de carcinoma de mama CD-E negativas no induce la expresión de CD-E. En cambio, estos autores observaron que la región 5' proximal del gen de CD-E humano muestra actividad específica en ensayos de transfección transitoria en líneas de carcinoma de mama, indicando que durante la progresión tumoral existen alteraciones de los factores que actúan en trans, que conducen a la inhibición de la expresión de CD-E.

En cualquier caso la metilación del DNA, no tiene porqué estar siempre relacionada con represión transcripcional, y puede jugar variados papeles en la carcinogénesis (Counts y Goodman, 1995). Así, en algunos casos la metilación puede favorecer la expresión génica por un mecanismo de "imprinting", como ocurre en el gen de Igf2r (Stöger *et al.*, 1993). Otras veces, la metilación de algunos residuos CpG previene la metilación de islas CpG próximas, como ocurre en un sitio de unión a SP1 del promotor del gen "housekeeping" aprt (Macleod *et al.*, 1994). Por otro lado, se ha demostrado que algunos factores, como SP1 (Höller *et al.*, 1988) o YY1 (Gaston y Fried, 1995), pueden unirse al DNA y activar la transcripción a pesar de que esté metilado. En este sentido, la metilación de la posición -56

del promotor de CD-E observada en el presente trabajo, que corresponde con la región rica en GC de unión a SP1 y AP2, en las líneas de queratinocitos analizadas (Fig. 33), no tiene porqué impedir la unión de dichos factores nucleares y de hecho esta posición se corresponde con un sitio de hipersensibilidad en los experimentos de footprinting *in vivo* (Fig. 34 y 35).

Por otro lado, los experimentos de footprinting *in vivo* (Fig. 34 y 35) muestran que los sitios de regulación postulados mediante ensayos *in vitro* o con un promotor exógeno están, en efecto, ocupados *in vivo* por factores, y esencialmente en células que expresan CD-E. El papel represor del elemento E-pal en células CD-E negativas se vería reflejado por la protección en estas líneas de la posición central de esta secuencia. Por otra parte, estos experimentos sugieren la existencia de otros posibles sitios reguladores. Destacan las posiciones -169/-167, -123/-126 y -117/-118, donde la existencia de sitios consenso de unión a factores Ets y SP1 hace pensar en la posibilidad de que estén actuando complejos mecanismos reguladores. Por otra parte, es sugerente la similitud de las secuencias protegidas -167/-169 y -117/-118 (GGAAGT).

Todos los resultados sobre el promotor de CD-E mostrados en esta Tesis, en conjunto, permiten hipotetizar sobre un modelo de regulación transcripcional de dicha molécula (Fig. 41). En células epiteliales, que expresan CD-E (Fig. 41A), factores de unión a la región GC (SP1 y AP2) y a la caja CCAAT, ejercerían un efecto transcripcional positivo, que estaría modulado por la interacción con factores tipo Ets. El factor de unión al elemento E-pal no sería capaz de bloquear la transcripción, probablemente debido a la naturaleza del complejo multitranscripcional formado por los otros factores.

Por otra parte, en células epiteloides o fusiformes, que representan estadíos más avanzados de la progresión, y son CD-E negativas, el papel del elemento represor E-pal sería el predominante. En estas líneas también existen factores tipo SP1 y AP2, pero no se unen a la región rica en GC de forma endógena, quizás por la existencia de factores Ets con diferentes propiedades a los de las líneas CD-E positivas.

2.3. Mecanismos de regulación comunes y diferenciales de los promotores de CD-E y CD-P.

Los promotores de CD-E y CD-P son muy similares en sus secuencias (ambos carecen de caja TATA y poseen una región rica en GC y una caja CCAAT) y comparten mecanismos de regulación. Sin embargo, los resultados obtenidos en este estudio sugieren que se regulan además por mecanismos diferentes. En concreto podrían intervenir factores comunes tipo SP1 y CP1, a través de la región rica en GC y la caja CCAAT de ambos promotores, respectivamente. Específicamente en la regulación del promotor de CD-E participaría el factor de transcripción AP2 y factores tipo CP2 y C/EBP, también a través de la región rica en GC y caja CCAAT, respectivamente.

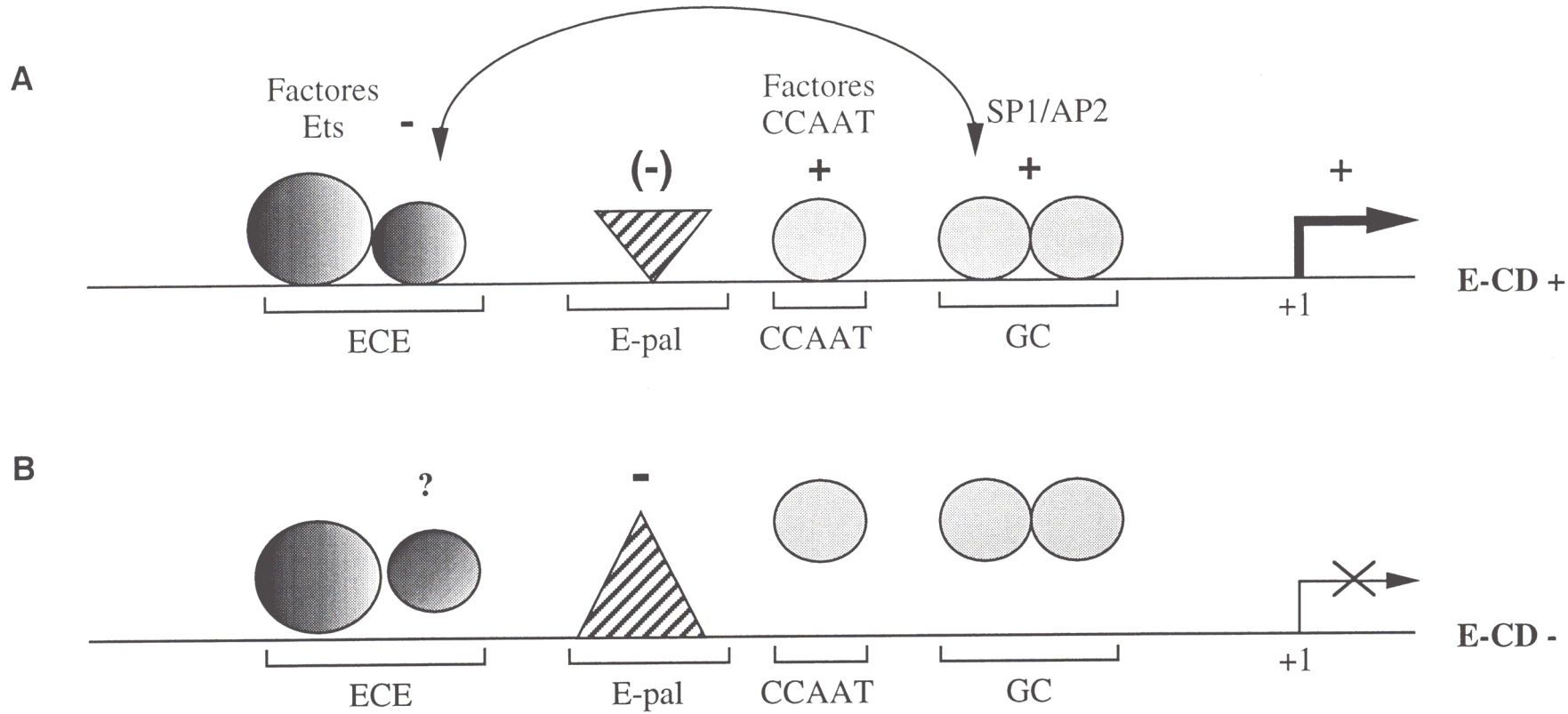

Figura 41. Modelo sobre la regulación del promotor de CD-E por factores nucleares. En la parte A de la figura se muestra un promotor de CD-E transcripcionalmente activo, mientras que en la parte B se muestra un promotor reprimido. Las figuras geométricas representan factores nucleares, que pueden unirse o no a la región promotora, con efecto activador (+) o represor (-). El modelo propuesto se ha elaborado a partir de los datos obtenidos en esta Tesis. Las posibles interacciones entre factores y los efectos que pueden tener se analizan en la Discusión con más detalle.

La diferente regulación de ambos promotores puede relacionarse con el diferente patrón de expresión que tienen ambos genes en la epidermis: CD-P restringida en la capa basal proliferativa y CD-E expresada en capas basales y suprabasales (Nose *et al.*, 1986; Fujita *et al.*, 1992).

La mayor complejidad de la regulación transcripcional de CD-E, con la existencia, por ejemplo, del elemento palindrómico E-pal, que tiene un papel represor predominante en células que no expresan CD-E, sugiere que este gen requiere una regulación muy fina, controlada por varios mecanismos. Además, los datos aportados en esta Tesis sugieren la participación de miembros de la familia Ets que formarían complejos transcripcionales capaces de modular la expresión de CD-E en los queratinocitos de ratón. En conjunto, todos estos resultados sugieren que no son los factores de transcripción aislados los que determinan el efecto transcripcional, sino la interacción de los factores entre sí y las interrelaciones que pueden establecer.

El conocimento de estos mecanismos puede ser de utilidad para intervenir en los procesos tumorales donde existe pérdida de CD-E que, como se ha indicado, puede contribuir a la adquisición de un fenotipo tumoral más agresivo. Por otra parte, los mecanismos de pérdida de expresión de CD-E en la carcinogénesis podrían ser comunes con los que ocurren durante el desarrollo embrionario, en las transiciones epitelio-mesénquima y su conocimiento puede aportar nuevos datos sobre el control de la expresión durante el desarrollo, así como sobre mecanismos de expresión epitelio específica.

CONCLUSIONES

1. El bloqueo de la expresión de CD-E en la línea celular de queratinocitos de ratón E24, que es tumorogénica pero no metastásica, induce en la misma un fenotipo invasivo y metastásico sin alterar los niveles de v-Ha-ras.

2. La CD-E controla negativamente la actividad de la gelatinasa MMP-9 en líneas de queratinocitos de piel de ratón. Este aumento en la actividad MMP-9 puede ser el responsable de la mayor capacidad invasiva y metastásica que poseen las células cuando pierden la CD-E.

3. La región -178/+92, respecto del sitio de inicio de la transcripción, del gen de CD-E de ratón presenta actividad transcripcional específica en líneas de queratinocitos de ratón.

4. La región rica en GC del promotor de CD-E (-58/-32), que une los factores nucleares SP1 y AP2, y la caja CCAAT (-65), que une factores relacionados con CP1, CP2 o C/EBP, juegan un papel regulador positivo en la transcripción de CD-E, y están ocupadas *in vivo* por factores nucleares solamente en las líneas de queratinocitos que expresan CD-E.

5. El elemento palindrómico E-pal (posiciones -86/-75) controla la transcripción de forma negativa, siendo el efecto represor mucho más fuerte en las líneas que no expresan CD-E. *In vivo* la posición central de este elemento está ocupada en todas las líneas de queratinocitos estudiadas.

6. El elemento de unión a Ets de la posición -95 regula negativamente la transcripción en aquellas líneas analizadas que expresan CD-E, posiblemente mediante la interacción con otros factores o cofactores nucleares. Endógenamente esta región está ocupada de forma diferente por líneas que expresan y no expresan CD-E.

7. La represión o disminución de la expresión de CD-E en líneas de queratinocitos de ratón no es debida a metilación endógena de la región 5' proximal del gen de CD-E.

8. La regulación transcripcional de los genes de CD-E y CD-P en queratinocitos de ratón comparte mecanismos similares: regulación por SP1 y CP1, a través de las regiones rica en GC y caja CCAAT de ambos promotores, respectivamente. Sin embargo, mecanismos adicionales actúan sobre el promotor de CD-E, sugiriendo que la regulación transcripcional de esta molécula es más compleja.

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ANEXO

Analysis of the E-Cadherin and P-Cadherin Promoters in Murine Keratinocyte Cell Lines From Different Stages of Mouse Skin Carcinogenesis

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We previously isolated the 5′ upstream sequences of the mouse P-cadherin gene, in which putative binding sites for several transcription factors were identified between nt −101 and +30. In the study reported here, the promoter activity of the postulated 5′ cis-acting sequences of the P-cadherin promoter, and the activity of the proximal E-cadherin promoter were investigated in several murine keratinocyte cell lines showing different levels of P- and E-cadherin expression as well as different morphology and tumorigenic behavior. Cell-type specificity and optimal activity of P-cadherin expression in murine keratinocytes was conferred by 5′ sequences located between nt −200 and +30, and the GC-rich region (nt −101 to +80) and a CCAAT box element (nt −65) had a major regulatory role. The cell-type specificity of the E-cadherin promoter, on the other hand, was mediated by a combination of positive regulatory elements, a GC-rich region (nt −58 to −24), and a CCAAT box (nt −65) and repressor elements inside the E-pal sequence. Interestingly, the maximum repressor effect of the E-pal element was observed in non-expressing undifferentiated spindle cells. In vitro binding studies indicated that the GC-rich region of the P-cadherin promoter was mainly recognized by Sp1-related nuclear factors, whereas both AP2- and Sp1-related factors were involved in the interaction of the GC-rich region of the E-cadherin promoter. Common factors (probably related to the CP1 family) seemed also to be involved in the recognition of the CCAAT-box element of both the E- and P-cadherin promoters, but additional specific factors participated in the interaction with the CCAAT box of the E-cadherin promoter. Our studies also support the hypothesis that loss or modification of some of the regulatory factors occurs during mouse skin tumor progression. *Mol. Carcinog. 20:33–47, 1997.* © 1997 Wiley-Liss, Inc.

Key words: cadherin expression; tumor progression; nuclear factors; regulatory elements

INTRODUCTION

Cadherins are major calcium-dependent cell-cell adhesion molecules whose involvement in morphogenetic and pathological processes has been extensively studied in the last decade [reviewed in 1,2]. It is presently assumed that cadherins constitute a gene superfamily in which four of the best characterized members, presently called "classical" cadherins [3], were first identified by their specific tissue distribution: E-cadherin (epithelial) [4–6], P-cadherin (placental) [7], N-cadherin (neural) [8], and liver cell adhesion molecule (L-CAM) (chicken liver) [9]. Although P-cadherin has the highest homology to E-cadherin (58% amino acid identity), both molecules exhibit a specific tissue distribution, with P-cadherin being highly expressed in some epithelial as well as non-epithelial tissues [10]. The differential expression of E- and P-cadherin is maintained even in some epithelia in which the two cadherins coexist: for instance, in the epidermis, E-cadherin is expressed in all living cells, including basal, spinous, and granular cells, whereas P-cadherin is restricted to the basal cell layer of the epidermis [7,11].

During embryonic development, cadherin expression occurs very early, and a specific spatio-temporal program for the different members must be completed for correct morphogenesis of the embryo [1,10]. In the adult organism, the specific expression of cadherins plays also an important role in the maintenance of tissue architecture. Interestingly, missexpression of cadherins occurs during the tumorigenic processes and may in part explain the disruption of cell-cell association that frequently takes place in tumor cells. Specifically, downregulation of E-cadherin has been reported in a high variety of

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Abbreviations: CAT, chloramphenicol acetyltransferase; L-CAM, liver cell adhesion molecule; SV40, simian virus 40.

human epithelial tumors and in animal models [reviewed in 2,12], and an anti-invasive role has been proposed for this molecule. In general, expression of E-cadherin in carcinomas is strongly correlated with the differentiation grade of the tumors [2]. In contrast, P-cadherin expression, although initially associated with the maintenance of the proliferative compartment in some tumors and normal epithelia [13,14], has also been related to the segregation of basal cell layers and the arrangement of epidermal cells in some skin appendages during embryonic development [11]. Our previous studies and those of other groups on E- and P-cadherin expression in mouse skin carcinogenesis indicated differential alterations in both molecules with tumor progression: downregulation of E-cadherin occurs in squamous cell carcinomas and in progressing papillomas, whereas increased expression of P-cadherin is observed in papillomas and squamous cell carcinomas, and both molecules are completely suppressed in fully undifferentiated spindle cell carcinomas [15–17].

To understand the regulation of cadherin expression during normal development and neoplasia, it is important to study the regulatory elements that control cadherin gene expression. The genomic organizations of E- [18], P- [19], and N-cadherin [20] and of L-CAM [21] were previously reported. Analyses of the E-cadherin promoter and regulatory elements have attracted great interest in recent years and have led to the identification of positive (GC-rich region and CCAAT box) and negative (E-pal) elements in the 5′ proximal sequences (nt −94 to +1) [22–25] and an epithelial enhancer in the first intron [25]. With the exception of L-CAM [26,27], much less is known about the regulation of the cadherin genes and in particular about the P-cadherin promoter. A previous study analyzed the role of distal 5′ sequences and of two large regions of the second intron of the P-cadherin gene in the transcriptional activity, without showing any apparent cell-type specificity [28]. We previously cloned about 1 kb of the 5′ sequence of the mouse P-cadherin gene and characterized the transcription start site and the first, small intron [29]. Sequence analysis showed that the 5′ proximal P-cadherin promoter region has some similarities to the mouse E-cadherin promoter, such as the absence of a TATA box and the presence of a CCAAT box (nt −65) and a GC-rich region (nt −101 to −80) containing potential binding sites for Sp1 and AP2 transcription factors, but they differ in the absence of a palindromic element (E-pal) in the P-cadherin promoter [29].

In the study reported here, we analyzed the involvement of the previously postulated 5′ proximal cis elements on the activity of the mouse P-cadherin promoter and the activity of the proximal E-cadherin promoter in several mouse skin carcinogenesis keratinocyte cell lines that exhibit different P- and E-cadherin expression levels, phenotypic behavior, and degrees of tumorigenicity [15]. The results indicated that the 5′ GC-rich sequences (which are recognized by Sp1-related factors) and the CCAAT regions of the P-cadherin promoter were involved in cell-type specificity and optimal activity. A more complex pattern of regulation existed in the E-cadherin promoter, involving positive regulatory elements (5′ GC-rich and CCAAT regions) and repressor elements (E-pal), the latter mainly acting in non-expression undifferentiated spindle carcinoma cells. Comparison of the GC-rich and CCAAT regions of the E- and P-cadherin promoters in murine keratinocytes also revealed the existence of common regulatory factors (Sp1 and CP1 related) in both promoters, of additional specific factors for the recognition of the E-cadherin promoter, and of involvement of AP2-related factors in the GC-rich region. Modification of some of the factors or alterations in their interaction with coactivators seemed to be involved in the downregulation of E- and P-cadherin expression in carcinoma-derived keratinocyte cell lines.

MATERIALS AND METHODS

Cell Culture

The origins of the mouse epidermal keratinocyte lines used here (MCA3D, PDV, HaCa4, and CarB) were previously described [15,30] (Table 1). The cells were routinely grown in Ham's F-12 medium supplemented with amino acids and 10% (v:v) fetal calf serum (GIBCO Ltd., Paisley, Scotland) at 37°C in a humidified 5% CO_2 atmosphere. For the transient transfection assays, this medium was replaced with Dulbecco's modified Eagle's medium (DMEM, GIBCO Ltd.) also supplemented with 10% fetal calf serum.

Plasmid Constructions

To obtain the different P-cadherin promoter constructs, appropriate fragments to be inserted into reporter plasmids were obtained from the plasmid pP5, a genomic clone containing P-cadherin nt −1060 to +3300 [29], after subcloning of the −1060/+285 SacI-SphI fragment and digestion with exonuclease III, which gave a common 3′ end to most of the fragments (nt +47), except for the ΔAP2 construct (see below). Digestion with suitable restriction enzymes made the 5′ ends of the different fragments at nt −200, −75, and −25. The fragments were inserted into the promoterless reporter plasmid pXP1 [31] upstream the luciferase gene. The ΔAP2 construct was obtained from the −200 construct by digestion with exonuclease III of nt +14 to +47 of the promoter. These different constructs are shown schematically in Figure 1A. The different E-cadherin promoter constructs, which contained several deletion fragments, the mutated E-pal sequence, or two independent subregions of the GC-rich region (GCI and GCII) of the E-cadherin promoter, were derived from the deletion construct −178/+92 cloned into the vector

Table 1. Characteristics of Epidermal Keratinocyte Cell Lines Used

Cell line	Origin	Morphology*	Expression†		Tumorigenicity‡
			E-cadherin	P-cadherin	
MCA3D	Primary epidermal culture treated with DMBA	E	+++	++	–
PDV	Primary epidermal culture treated with DMBA	E	++	+++	+
HaCa4	Derived from a squamous cell carcinoma	Ed	–	++	++
CarB	Derived from a spindle cell carcinoma	F	–	–	++

*E, epithelial; Ed, epithelioid; F, fibroblastoid.
†Relative levels estimated by northern blot analysis [15].
‡Tumorigenic behavior was analyzed by injection into nu/nu mice and estimated from the number of tumors and latency periods [15,30].

pCAT basic and were previously described in detail [22,25]. The E-pal sequence was mutated to CACCTTTAGGTG and the GCI and GCII subregions were mutated to GCCGTTTTC and TTCTGCGGG, respectively [25]. The construct containing a deleted E-pal sequence (Δ-Epal) was obtained from the deletion construct –178/+92 by removal of the nt –99 to –79 PstI fragment, blunt ended with T4 DNA polymerase, and religated. The various E-cadherin promoter constructs used are schematically represented in Figure 2A and Table 2. The hybrid GC.E/–75 promoter was obtained by introduction of a double-stranded oligonucleotide containing the complete GC-rich region from the E-cadherin promoter (nt –60 to –23) into the –75 construct of the P-cadherin promoter by using the 5′ BamHI restriction site. The hybrid E-pal/–200 construct was obtained by insertion of a double-stranded oligonucleotide containing the E-pal sequence from the E-cadherin promoter (nt –92 to –69) in the XhoI restriction site located upstream of the –200 construct of the P-cadherin promoter.

Transient Transfection Assays

The Ham's F-12 medium, in which cells were growing at about 25% confluence, was replaced with Dulbecco's modified Eagle's medium 4–6 h before transfection. Cells in 6-cm cell-culture dishes were transfected by the calcium phosphate precipitation method with 5 μg of the different luciferase or chloramphenicol acetyltransferase (CAT) reporter constructs. In the P-cadherin promoter analysis, transfection efficiency was monitored by cotransfection with 4 μg of the plasmid pCH110 (Promega Corp., Madison, WI), which contains the *Escherichia coli lacZ* gene under the control of the simian virus 40 (SV40) promoter or the plasmid RSV-CAT [32], which contains the reporter gene for CAT under the control of the Rous sarcoma virus promoter. For the E-cadherin promoter analysis, transfection efficiency was monitored by cotransfection with 2.5 μg of CMV-Luc plasmid which contains the reporter luciferase gene under the control of the cytomegalovirus promoter [33]. Sixteen hours after the addition of the precipitate, the cells were washed with phosphate-buffered saline, and the medium was replaced with fresh Ham's F-12. After an additional 24 h, the cells were harvested and resuspended in 0.25M Tris HCl, pH 7.5, and cell extracts were obtained by three consecutive freeze-and-thaw cycles. For P-cadherin promoter transfections, β-galactosidase activity (in 50 μL of cell extracts) or CAT activity (in aliquots containing 20 μg of protein) were first determined as described previously [34,35], and cell-extract aliquots with equivalent β-galactosidase or CAT activities were then assayed for luciferase activity with a kit (Promega Corp.). The activities driven by the different P-cadherin promoter constructs were compared with that produced by the CMV-Luc reporter plasmid in the different cell lines and expressed as the percentage of CMV-Luc activity. The basal activity of the promoterless pXP1 plasmid was also determined. For E-cadherin promoter transfections, the luciferase activity of cell extracts were first analyzed as described above, and aliquots containing equivalent luciferase activity were then analyzed for CAT activity as described previously [35]. The activities driven by the different E-cadherin promoter constructs were compared with that produced by the pCAT-control vector, which contains the *CAT* gene under the control of the SV40 promoter, in the different cell lines and expressed as the percentage of SV40-CAT activity. The basal activity of the promoterless pCAT-basic vector (Promega Corp.) was also determined and subtracted.

Gel Retardation and In Vitro Footprinting Assays

Nuclear extracts were prepared from the different keratinocyte cell lines as previously reported for HeLa cells [36]. Gel retardation assays were performed basically as previously described [37], with some modifications in the DNA-protein incubation buffer (which was 20 mM HEPES, pH 7.9; 60 mM KCl; 10% glycerol; 0.1 mM EDTA; 2 mM $MgCl_2$; and 1 mM dithiothreitol). As a nonspecific competitor, 1 μg of poly(dI-dC) (Boehringer Mannheim S.A., Barcelona, Spain) was used. For supershift analysis, anti-Sp1 and

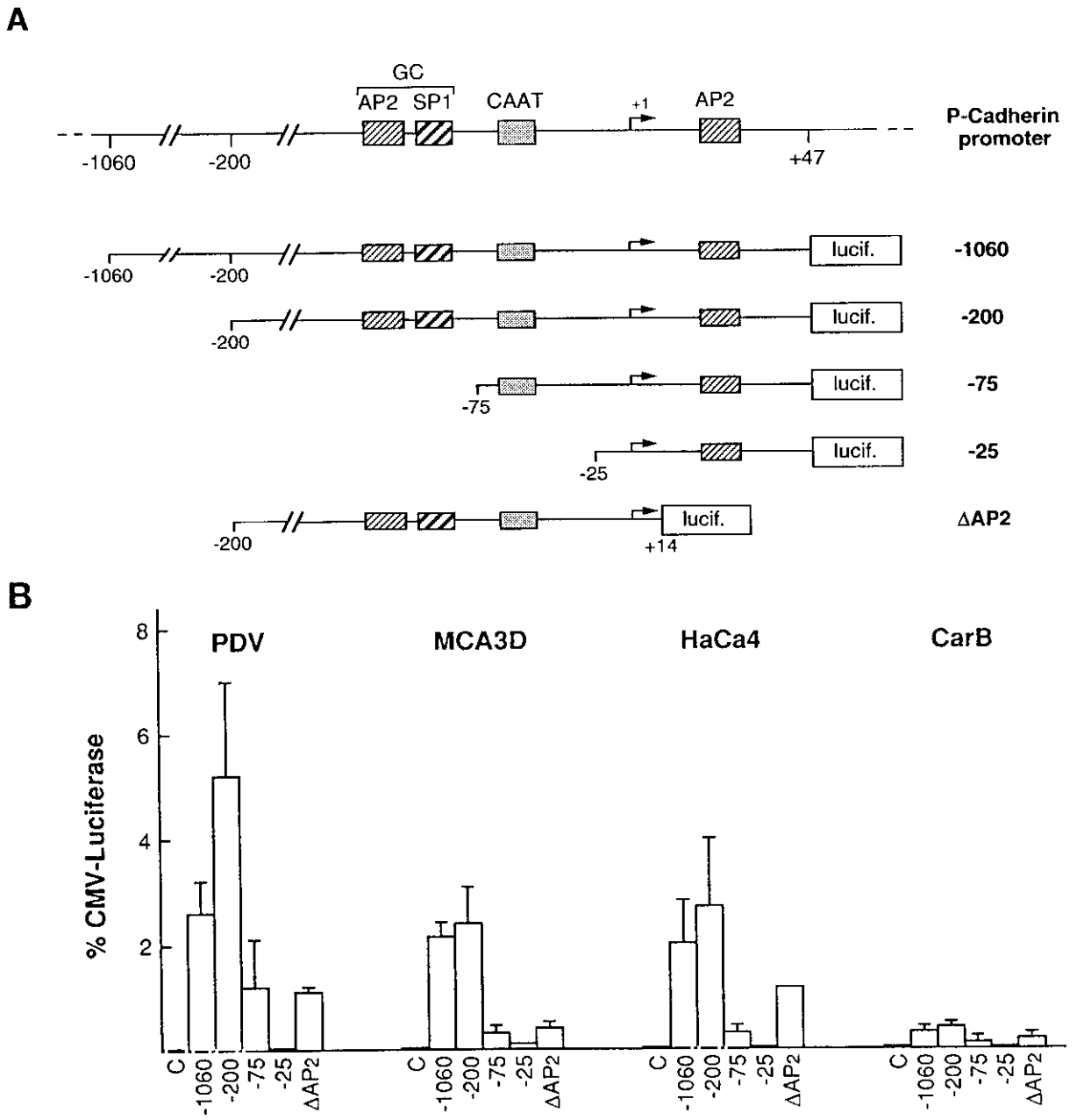

Figure 1. (A) Schematic representation of the different constructs obtained with deletion fragments of the P-cadherin 5′ upstream region (–1060, –200, –75, –25, and ΔAP2) fused to a luciferase reporter gene in the pXP1 promoterless vector as described in Materials and Methods. Shown are potential binding sites for the transcription factors Sp1 (▨), and AP2 (▨) and the CCAAT box (■). The arrow shows the transcription initiation site. (B) Promoter activity of the different constructs of the P-cadherin promoter in the indicated keratinocyte cell lines. The luciferase activity of the different deletion constructs was normalized to β-galactosidase or CAT activity in the same extract and is represented as the percentage of the activity driven by the cytomegalovirus promoter (CMV-Luc) in each cell line. The various P-cadherin deletion constructs are indicated below the bars; luciferase activity driven by plasmid pXP1 (C) is also represented. The results shown are average values from three to 10 independent experiments performed in duplicate. The error bars indicate standard deviations.

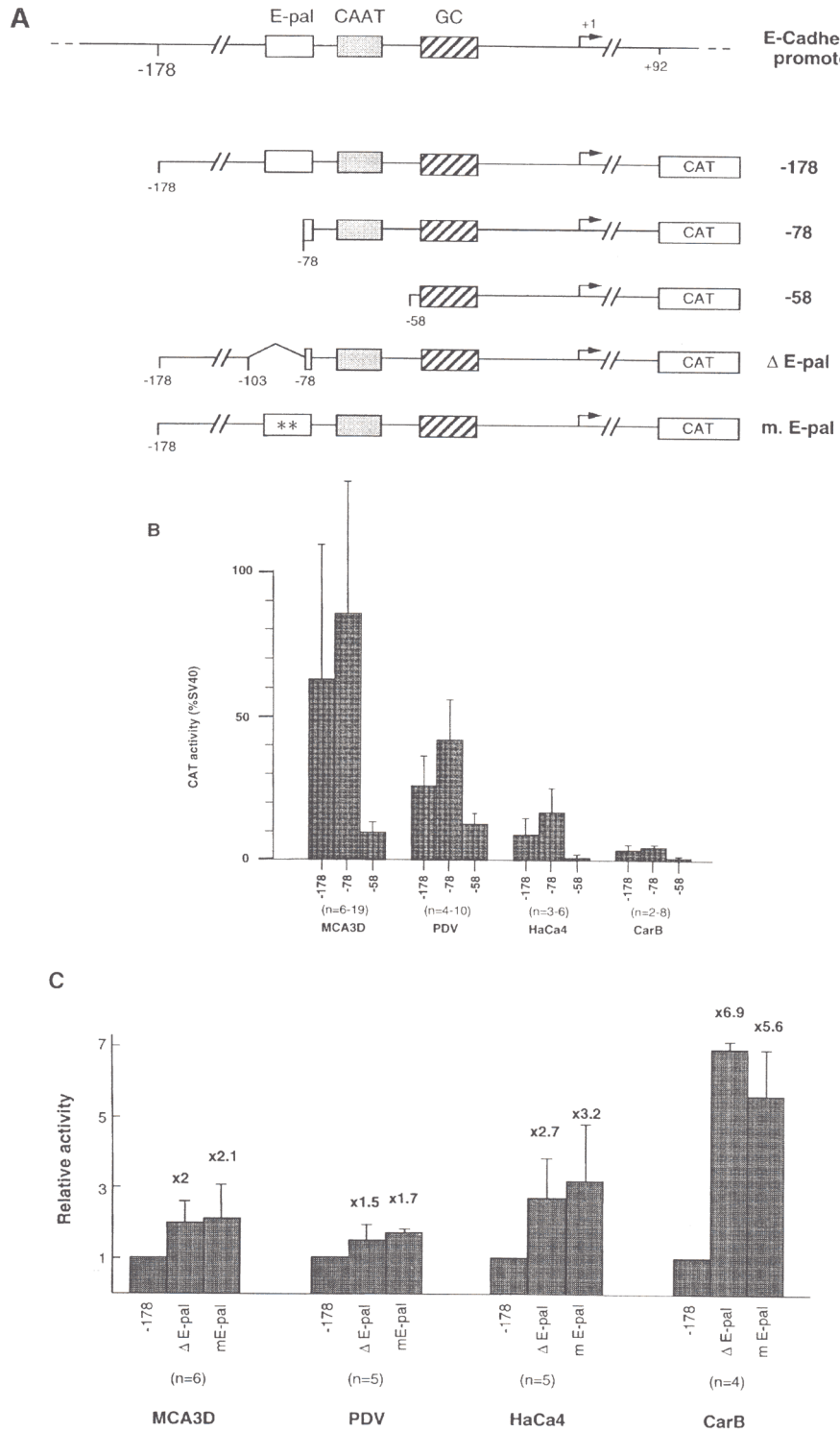

Figure 2. (A) Schematic representation of the different constructs obtained with deletion fragments of the E-cadherin 5′ upstream region (–178, –78, –58, ΔE-pal, mE-pal) fused to a *CAT* reporter gene in the pCAT basic promoterless vector as described in Materials and Methods. Shown are transcription-factor binding sites E-pal (□), the GC-rich region (▨) and the CCAAT box (▩). The arrow shows the transcription initiation site. (B) Promoter activity of the different constructs of the E-cadherin promoter in the indicated keratinocyte cell lines. The CAT activity of each deletion construct was normalized to luciferase activity in the same extract and is represented as the percentage of the activity driven by the SV40 promoter (pCAT control vector) in each cell line. The various E-cadherin deletion constructs are indicated below the bars, as is the number of independent experiments (n) performed in duplicate. The results shown are the average values, and the error bars show the standard deviations. (C) Promoter activity of the E-cadherin constructs containing mutated or deleted E-pal elements in the different cell lines. The CAT activities were measured as described in panel B and normalized to the relative activity of the –178 construct in each cell line. The number of independent experiments (n) performed in duplicate is indicated. The values are averages and the error bars indicate the standard deviations. The fold-increase in promoter activity obtained in each case is indicated by the numbers on the top of the error bars.

Table 2. Effects of Mutations in the CG-Rich Region on E-cadherin Promoter Activity

Promoter constructs†	Relative activity (±SD)* MCA3D (n=3)	HaCa4 (n=4)
Wild type (–178) GCCGGGGGCGGTGCCTGCGGGC	1	1
Mutant GCI GCCGTTTTCGGTGCCTGCGGGC	0.4 (0.14)	0.44 (0.07)
Mutant GCII GCCGGGGGCGGTTTCTGCGGGC	0.52 (0.04)	0.63 (0.11)

*The activity of the E-cadherin promoter, estimated as described in Figure 2, is expressed relative to the –178 construct in each cell line. n, number of independent experiments performed in duplicate.
†Constructs containing nt –178 to +92 of the E-cadherin promoter were analyzed. The sequence of the GC-rich region (nt –53 to –32) of the wild-type and GCI and GCII mutants is indicated.

anti-AP2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) or control rabbit IgG (1 μg) were added to the reaction mixtures and incubated for 25 min at room temperature. The following oligonucleotides, which correspond to different elements of the E- and P-cadherin promoters [22,29] and their complements were used: CCAAT.P (GTTGGACCAATCAGCAGC), CCAAT.E (CGTCCCCAGCCAATCAGC), GC.P (TCGACCCCACCTGCGAGGGGGCGGGACC), GC.E (CAGCGGCGCCGGGGGCGGTGCCTGCGGGCTCACCTGGC), E-pal (GGCTGCCACCTGCAGGTGCGTCCC), SP1.P (CGAGGGGGCGGGACCTTG), and AP2.P (GCCTCGACCCCACCTGCG).

The oligonucleotides containing the consensus binding sequences of different transcription factors used as competitors were Sp1.SV40 [38], AP2.SV40 [39], CP1 and CP2 [40], C/EBP [41], and NF1 [42]. All oligonucleotides used contained BamHI overhangs at their 5′ and 3′ ends (GATCC and G, respectively). Complementary oligonucleotides were hybridyzed, and the double-stranded products were end labeled with the Klenow fragment of DNA polymerase [34].

For DNAse I footprinting analysis of the P-cadherin promoter, the nt –200 to +120 KpnI-KpnI fragment was labeled at the 3′ end of the noncoding or coding strand with Klenow fragment. For DNAse I footprinting analysis of the E-cadherin promoter, an XbaI-KspI fragment containing nt –178 to +17 was labeled at the 3′ end of the noncoding strand with Klenow fragment. Footprinting reactions were performed as described previously [37] by using 10–50 μg of nuclear extracts, 1 μg of poly(dI-dC), and 1–2 ng (about 15 000 cpm) of labeled probe per reaction. Sp1 footprinting assays were performed with recombinant purified human transcription factors (Promega Corp.) (1 foot printing unit (fpu)), and no poly(dI-dC) was added.

RESULTS

Promoter Activity of the 5′ Flanking Regions of the Mouse P-cadherin and E-cadherin Genes in Mouse Keratinocyte

Our previous study of 5′ sequences of the mouse P-cadherin gene revealed several putative binding sites for transcription factors in the proximal region: a GC-rich region (nt –101 to –80) containing potential binding sites for Sp1 (nt –85) and AP2 (nt –95), a CCAAT box (nt –65), and an additional AP2 binding site downstream of the initiation site (nt +30) [29]. Comparison with the 5′ sequence of the mouse E-cadherin gene showed that similar regulatory sequences were present in the mouse E-cadherin promoter region: a CCAAT box (nt –65) and a GC-rich region (nt –58 to –25) with binding sites for AP2 and Sp1 factors. However, the 5′ sequences differed in that the E-cadherin gene has a palindromic E-pal element (nt –86 to –75) [22].

To investigate the involvement of the postulated 5′ regulatory elements in the activity of both cadherin promoters in normal and malignant keratinocytes, transient transfection assays were performed with four selected murine keratinocyte cell lines previously characterized for their expression of E- and P-cadherin [15]. As summarized in Table 1, the cell lines also varied in their tumorigenic behavior and morphological phenotypes. The P-cadherin levels ranged from nil in undifferentiated CarB cells to the highest level in epithelial PDV cells (PDV > HaCa4 ≥ MCA3D; CarB, negative). Regarding E-cadherin expression, two of the cell lines were positive (MCA3D > PDV), and the other two (HaCa4 and CarB) were negative. To analyze the activity of the P-cadherin promoter, deletion fragments of the 5′ region containing the different putative regulatory elements were fused to a luciferase reporter gene (Figure 1A) and tested in transfection assays with the selected cell lines. As can be observed in Figure 1B, the longest construct, –1060, exhibited similar activity in the three P-cadherin–expressing cell lines (PDV, MCA3D, and HaCa4) and very low activity in non-expressing CarB cells (about 10% of that of PDV cells). The –200 construct, which contained the putative binding sites for Sp1 and AP2 (GC-rich region), AP2 (nt +30), and CCAAT binding proteins, showed the highest activity in all cell lines tested. The activity of the –200 construct closely resembled the level of P-cadherin mRNA expression in the four keratinocyte cell lines, being stronger in PDV cells than in HaCa4 and MCA3D cells and very weak in CarB cells (about 12-fold lower than in PDV).

Deletion of nt –200 to –76 which contains the 5′ AP2 and Sp1 putative binding sites (construct –75) led to a strong decrease in the promoter activity in all the cell lines (to 11–22% of that of the –200 construct). The promoter activity further dropped to nearly the level of the pXP1 promoterless control

vector when the region containing a CCAAT-box sequence was deleted (–25 construct). When the second putative AP2 binding site, located at nt +30, and its flanking sequences were removed from the –200 fragment (ΔAP2 construct), a decrease of variable intensity (to 40–80% of that of the –200 construct) was observed in the different cell lines (Figure 1B).

The activities of the –1060 and –200 constructs were also tested in three additional P-cadherin–negative cell lines: NIH3T3 fibroblasts, which are negative for P-cadherin expression [28], and two independent spindle carcinoma cell lines, MSC11A5 and MSC11D3 [43], which express very low levels of P-cadherin mRNA (about 5% of that of PDV cells). The –200 construct exhibited a low promoter activity (0.3–0.8% of that of CMV-luc activity) in the three cell lines, and the relative activity of the –1060 construct was similar to that observed in CarB cells (data not shown).

These results indicated that elements conferring cell-type specificity and optimal activity for P-cadherin expression in cultured keratinocytes appeared to be within nt –200 to +30 of the mouse P-cadherin gene and suggested that the GC-rich region and the CCAAT box can play a regulatory role.

The activity of the E-cadherin promoter in the keratinocyte cell system was also analyzed by transient transfection assays using several constructs containing different elements of nt –178 to +92 of the mouse E-cadherin gene coupled to a *CAT* reporter gene (Figure 2A) [22,25]. As shown in Figure 2B, the 5′ cis elements present in the –178 construct of the E-cadherin promoter exhibited cell-type specificity, as this construct was active in expressing cells, with the highest activity detected in MCA3D, less activity in PDV, and almost no activity in non-expressing CarB cells. In contrast, the –178 promoter construct had moderate activity in non-expressing HaCa4 cells. Similar results were obtained when a longer fragment up to nt –1400 of the E-cadherin gene was used (data not shown). Deletion of distal 5′ sequences containing the E-pal element (–78 construct) gave rise to a significant increase in the promoter activity in all tested keratinocyte cell lines, whereas additional deletion of the CCAAT box (–58 construct) partly (MCA3D and PDV) or completely inhibited (HaCa4) the promoter activity. To analyze the role of the E-pal element, two additional constructs in which the central two nucleotides of the E-pal sequence were either mutated (m-E-pal) or deleted (ΔE-pal) from the –178 construct were used (Figure 2A). The mutated E-pal element induced a moderate to strong increase in the activity of the E-cadherin promoter in the four cell lines, with a higher effect observed in the non-expressing HaCa4 cells (about a threefold increase) and more significantly in spindle CarB cells (sixfold to sevenfold increase) (Figure 2C). The role of the GC-rich region was also analyzed by using two additional constructs in which two subregions (GCI and GCII) containing potential binding sites for AP2 and Sp1 factors were mutated [25]. As shown in Table 2, mutation of both regions decreased the E-cadherin promoter activity 60% and 40–50% for GCI and GCII mutants, respectively, both in MCA3D and HaCa4 cells, as previously reported in other cell types [22,25]. On the other hand, the promoter activity of a construct (–21/+92) in which the GC-rich region was also deleted was even less than that of the –58 construct in all tested cell lines (data not shown).

Taken together, the analysis of the E-cadherin promoter indicated that the cell-type specificity in cultured keratinocytes was controlled by positive elements (the GC-rich and CCAAT regions) and repressor elements (the E-pal sequence). Interestingly, the repressor activity was strong in undifferentiated spindle CarB cells.

Binding of Nuclear Factors to the E- and P-Cadherin Promoters

To begin to study the specific sequences and nuclear factors involved in the recognition of the proximal P- and E-cadherin promoters, we performed DNAse I footprinting assays. Analysis with nt –200 to +120 of the P-cadherin promoter showed that nuclear extracts from CarB, HaCa4, and PDV cells protected only a wide region from nt –98 to –60 (Figure 3A). Similar results were obtained when the coding strand was used as a labeled probe and with MCA3D nuclear extracts (data not shown). The protected area clearly covered the CCAAT box (nt –64 to –60) and part of the GC-rich region, including the complete Sp1 binding site (nt –88 to –80) and the 3′ half of the AP2 binding site (nt –98 to –95). On the other hand, no protection was observed at any other region in nt –200 to +120, including the distal 5′ upstream sequences, the transcription start region, and the 3′ AP2 site at nt +30. Incubation of the probe with purified Sp1 factor gave rise to a narrower protection pattern (nt –98 to –74) (Figure 3A, lane Sp1), indicating the participation of a Sp1-related factor in the recognition of that region and the presence in the nuclear extracts of additional factors able to bind to the nt –73 to –60 protected region. Evidence for involvement of a Sp1-related factor in the recognition of the nt –94 to –74 region was obtained by competition experiments with an oligonucleotide containing a consensus Sp1 binding site from the SV40 promoter. This oligonucleotide was able to totally compete the protection of the nt –98 to –74 region generated by purified Sp1 factor or PDV nuclear extract but only partially compete the nt –73 to –60 region protected by PDV nuclear factors (Figure 3B, + lanes). In contrast, no protection of nt –98 to –60 was observed with purified AP2 factor; nor were consensus AP2 oligonucleotides able to compete the protected region (data not shown). These results strongly suggested that a Sp1-related

factor is involved in the recognition of the nt –94 to –74 GC-rich region of the P-cadherin promoter. The same protected pattern was observed in expressing (PDV, HaCa4, and MCA3D) and non-expressing (CarB) keratinocyte cell lines.

Footprinting analysis of the proximal E-cadherin promoter (nt –178 to +17) was also performed with nuclear extracts from E-cadherin–positive (MCA3D) and –negative (HaCa4 and CarB) cell lines (Figure 3C). Three protected regions were detected in expressing MCA3D cells: (a) the proximal GC-rich and CCAAT regions, (b) the 3′ end of the E-pal element, and (c) the 5′ end of the E-pal and additional 5′ upstream sequences, including a second GC-rich region. A hypersensitive site in the center of the E-pal element was also detected in MCA3D extracts. A similar protection pattern was detected in other E-cadherin–positive cells, like PDV (data not shown). In contrast, the protection pattern obtained with nuclear extracts from HaCa4 and CarB cells showed a weaker or almost complete lack of protection of the proximal GC-rich/CCAAT region (a) and of the 5′ distal sequences (c). In addition, there was weaker protection of the 3′ end of the E-pal element and absence of the hypersensitive site in both HaCa4 and CarB extracts. These results suggested that loss or modification of some of the nuclear factors involved in the recognition of the proximal E-cadherin promoter occurred in non-expressing keratinocytes.

The binding sites, included in the CCAAT box and GC-rich region of both the E- and P-cadherin promoters, were further analyzed by gel retardation assays using nuclear extracts from the different cell lines. To study the factors recognizing the CCAAT box element in both promoters, gel retardation assays were performed with labeled CCAAT oligonucleotides from the E- and P-cadherin promoters. As shown in Figure 4A, when PDV nuclear extracts were used, three specific retarded complexes were obtained with the E-cadherin CCAAT probe (CCAAT.E), whereas only one specific retarded complex (exhibiting the same apparent mobility as but a stronger intensity than that of the slower-migrating complex

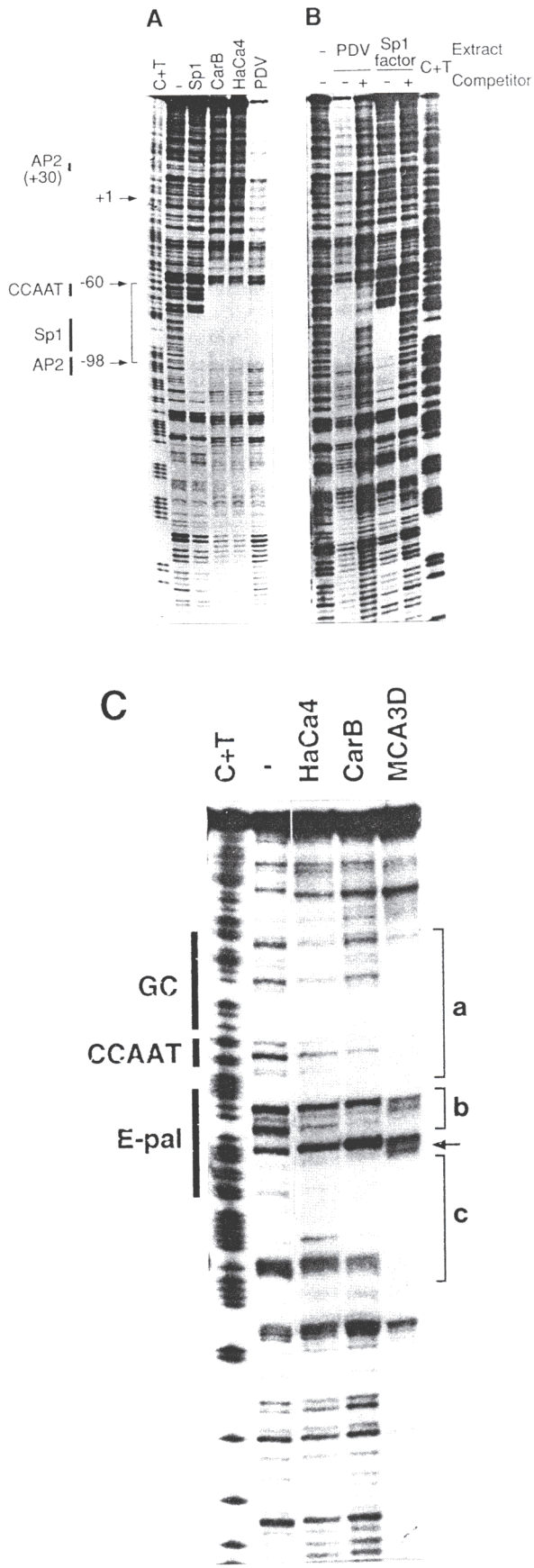

Figure 3. DNAse I footprinting analysis of the 5′ proximal P- and E-cadherin promoters. (A and B) Analysis of the P-cadherin promoter. The nt –200 to +120 fragment of P-cadherin 3′ end labeled with ^{32}P on the noncoding strand was incubated in the absence (–) or presence of Sp1 purified transcription factor (1 foot printing unit) and 4 ng of DNAse I or nuclear extracts of the indicated cell lines (CarB, 43.5 μg; HaCa4, 47 μg; PDV, 10.5 μg) and 40 ng of DNAse I. Panel B shows footprinting protection of the P-cadherin promoter with PDV nuclear extracts or Sp1 purified factor in the absence (–) or presence (+) of 50 ng of an oligonucleotide containing the Sp1 binding site of the SV40 promoter. (C) Analysis of the E-cadherin promoter. The nt –178 to +17 fragment of E-cadherin 3′ end labeled with ^{32}P on the noncoding strand was incubated in the absence (–) or presence of nuclear extracts of the indicated cell lines (HaCa4, 47 μg; CarB, 53 μg; MCA3D, 60 μg) and 40 ng of DNAse I. The main areas protected by the nuclear extracts (brackets), the sequence binding sites for the different factors (thick bars) and the transcription start site (nt +1), are indicated. C + T, products of Maxam-Gilbert sequencing reactions to the probes.

detected with the CCAAT.E probe) was obtained with the P-cadherin CCAAT probe (CCAAT.P). An excess of cold CCAAT.P oligonucleotide competed exclusively the slower-migrating complex of CCAAT.E, without affecting the other two complexes. Conversely, an excess of cold CCAAT.E oligonucleotide partially competed the specific retarded complex detected with the CCAAT.P probe. Oligonucleotides containing consensus binding sites for the CCAAT-binding factor CP1 [40] partially competed the complex obtained with the CCAAT.P probe and competed effectively the three complexes generated by the CCAAT.E probe, which were also partially competed by oligonucleotides containing consensus binding sites for the CCAAT-binding factor CP2. On the other hand, oligonucleotides containing consensus binding sites for C/EBP factor [41] effectively competed the two faster-migrating complexes generated by the CCAAT.E probe without affecting the slower one or the complex obtained with the CCAAT.P probe. No competition of the complexes obtained with either probe was detected when oligonucleotides containing consensus binding sites for NF1 factor [42] were used. Identical results were obtained when nuclear extracts from HaCa4 and CarB cells were analyzed (data not shown). These results indicate that common nuclear factors, probably related to the CP1 family, participated in the recognition of the CCAAT box of both E- and P-cadherin promoters and that additional specific factors related to the CP2 and CAAT/Enhancer Binding Protein (C/EBP) families were also involved in the CCAAT-box interaction of the E-cadherin promoter in murine keratinocytes.

We also analyzed the proximal GC-rich regions of both promoters by band-shift assays using the corresponding GC-rich oligonucleotides as labeled probes. The results obtained with PDV nuclear extracts are shown in Figure 4B. The proximal GC-rich region from the E-cadherin promoter (GC.E) gave rise to three specific retarded complexes: two (complexes 1 and 2) with the same apparent mobility of those obtained with the GC-rich region of the P-cadherin promoter (GC.P) and an additional complex of intermediate mobility (complex 3). The two complexes obtained with the GC.P probe, which exhibited a stronger intensity than those obtained with the GC.E probe, were effectively competed with an excess of cold GC.P and GC.E oligonucleotides. In contrast, an excess of cold GC.P oligonucleotide, although competing efficiently the complexes 1 and 2, was unable to compete the specific mobility complex 3 generated by the GC.E probe. The two complexes of GC.P probe were also competed with oligonucleotides containing the Sp1 binding site from the P-cadherin promoter (SP1.P) and consensus Sp1 oligonucleotides from the SV40 promoter (SP1.SV40) but were only weakly competed by oligonucleotides containing AP2 binding sites from the P-cadherin (AP2.P) or SV40 (AP2.SV40) promoters. On the other hand, the three complexes generated with the GC.E probe were competed by oligonucleotides containing consensus AP2 binding sites from the SV40 promoter (AP2.SV40), but only weak competition of the GC.E complexes was detected with oligonucleotides containing the AP2 binding site from the GC-rich region of the P-cadherin promoter (AP2.P). In addition, the complexes 1 and 2 obtained with the GC.E probe were competed with oligonucleotides containing consensus Sp1 binding sites from the SV40 promoter (SP1.SV40) and from the GC-rich region of the P-cadherin promoter (SP1.P), but both types of Sp1 oligonucleotides were unable to compete complex 3.

Band-shift analysis of the GC-rich region of both promoters was also performed with nuclear extracts from HaCa4 cells (E-cadherin–/P-cadherin+) and CarB (E-cadherin–/P-cadherin–), with the same competitors as for PDV extracts. The results, presented in Figure 5, showed that the retardation patterns of the E- and P-cadherin GC-rich regions obtained with both nuclear extracts were the same as for PDV cells. The full set of competitions performed with HaCa4 extracts is presented in Figure 5A. As can be observed, competition of the two retarded complexes of the P-cadherin region was the same as that obtained with PDV extracts: competition by SP1.P and SP1.SV40 oligonucleotides and no competition with AP2.P and consensus AP2.SV40 oligonucleotides. However, competition of the three retarded complexes of the E-cadherin region was somewhat different than the results obtained with PDV extracts, as no competition of complexes 1 and 2 of the GC.E region was detected with AP2.P or consensus AP2.SV40 oligonucleotides (compare Figure 5A with 4B), although these two complexes were effectively competed by GC.P, SP1.P, and SP1.SV40 oligonucleotides. The competition patterns obtained with CarB extracts with both E- and P-cadherin regions were similar to those obtained with HaCa4 extracts (Figure 5B and data not shown).

The involvement of Sp1 factor in the complexes of the GC-rich regions of both promoters was confirmed by supershift analysis. As can be observed in Figure 6, anti-Sp1 antibodies supershifted the complexes generated by the GC-rich regions of both promoters in nuclear extracts from PDV, HaCa4, and CarB cells. In all cases, the supershifted band was associated with a decrease in the faster-mobility complex of the GC-rich regions of both promoters. Anti-AP2 antibody did not supershift any of the complexes obtained with the GC-rich region of the P-cadherin promoter; however, it induced a decrease in the specific E-cadherin complex 3 (in PDV and CarB extracts) and the appearance of a supershifted band (in HaCa4 extracts).

These results indicated that the GC-rich region of the P-cadherin promoter was mainly recognized by Sp1-related nuclear factors, whereas the corresponding region of the E-cadherin promoter exhibited a

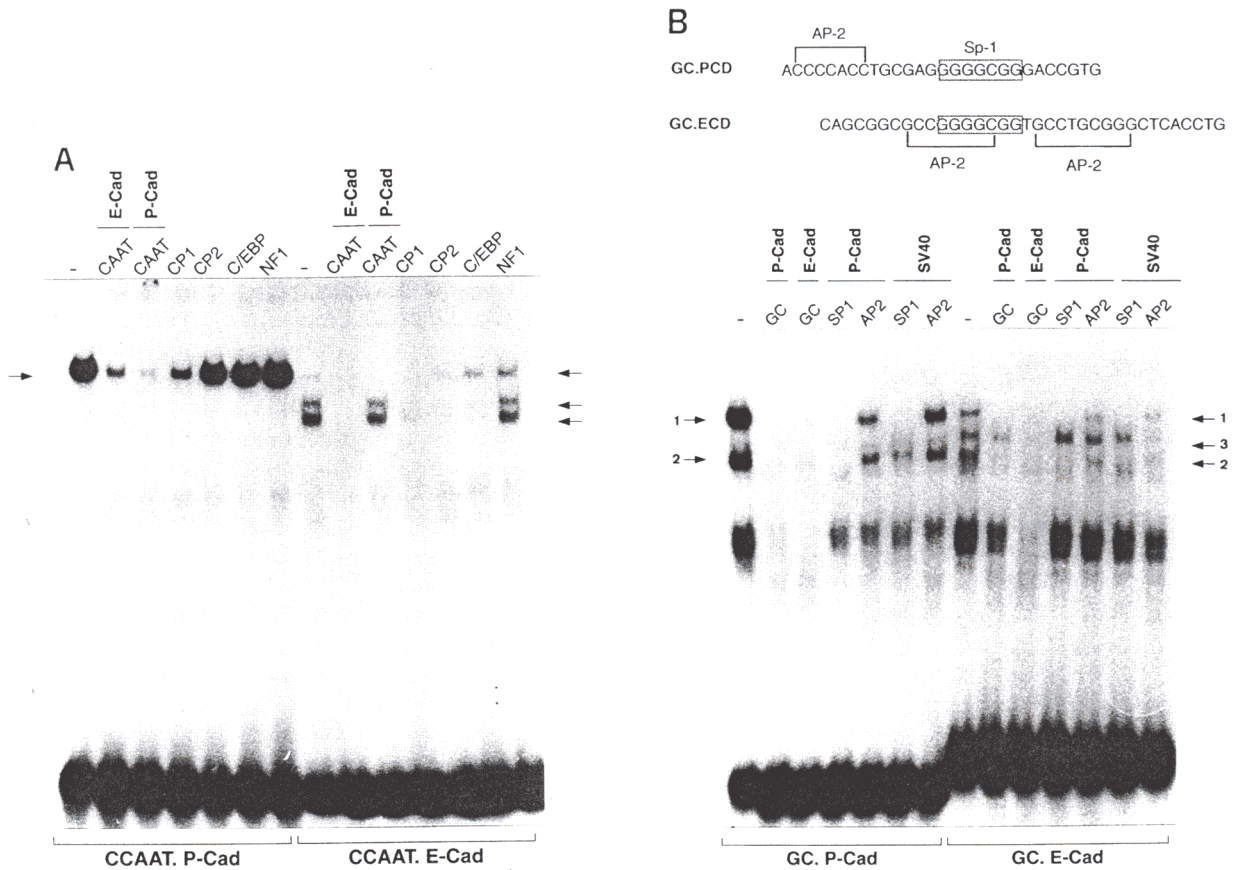

Figure 4. Band-shift analysis of the CCAAT box and GC-rich regions of E- and P-cadherin promoters with PDV extracts. Gel retardation assays were performed with 4 μg of PDV nuclear extracts, as indicated in Material and Methods. (A) Labeled oligonucleotides containing the CCAAT box from the E-cadherin (CCAAT.E) and P-cadherin (CCAAT.P) promoters used as probes. Cold oligonucleotides containing consensus sites for different CCAAT binding factors (CP1, CP2, C/EBP, and NF1) or cold E- and P-cadherin CCAAT oligonucleotides were used as competitors with a hundredfold molar excess of the labeled probes. (–), no competitor added. The main retarded complexes are indicated by arrows. (B) The sequences containing the GC-rich region from the E-cadherin (GC.ECD) or P-cadherin (GC.PCD) promoters, indicated at the top, were used as labeled probes in the absence (–) or presence of a hundredfold molar excess of cold oligonucleotides. The competitor oligonucleotides used were cold GC.E and GC.P oligonucleotides and oligonucleotides containing Sp1 or AP2 binding sites from the P-cadherin promoter or the SV40 promoter, as indicated above the lanes. The main retarded complexes observed with the GC.E probe are indicated by arrows on the right (complexes 1, 2, and 3), and those obtained with the GC.P probe are indicated on the left (complexes 1 and 2). The positions of potential Sp1- and AP2-binding sites in the GC.PCD and GC.ECD regions are shown at the top of the panel.

more complex pattern, with the involvement of Sp1-related factors and the additional participation of AP2-related factors in murine keratinocytes. The results also support the existence of differential modifications (or specific isoforms) of those factors in E-cadherin –expressing and –non-expressing keratinocytes.

On the other hand, band-shift analysis of the E-pal region of E-cadherin showed the presence of one specific retarded complex in both expressing and non-expressing keratinocytes (data not shown), similar to previous observations in other cell systems [22,25]. Finally, there was no evidence for direct involvement of the second AP2 site of the P-cadherin promoter located downstream of the transcription start site, as no specific retarded complexes were detected when a labeled oligonucleotide containing the AP2 binding sequence at nt +30 was incubated with nuclear extracts from any cell line.

Influence of the GC-Rich and E-Pal Regions of E-Cadherin Promoter in the Activity of the P-Cadherin Promoter in Epidermal Keratinocytes

The similarities and differences detected in the sequence analysis and in the band-shift assays of the GC-rich regions of the E- and P-cadherin promoters raised the question of whether the regions were interchangeable. Therefore, we designed a hybrid promoter construction in which the GC-rich region of the P-cadherin promoter was replaced by that of the E-cadherin promoter by inserting the GC.E region (nt –60 to –23) 5′ of the –75 P-cadherin construct, which had the endogenous GC-rich region deleted. The activity of this construct was tested in the different keratinocyte cell lines in parallel with the –200 and –75 P-cadherin constructs. As shown in Table 3, the GC.E-rich region moderately increased the ac-

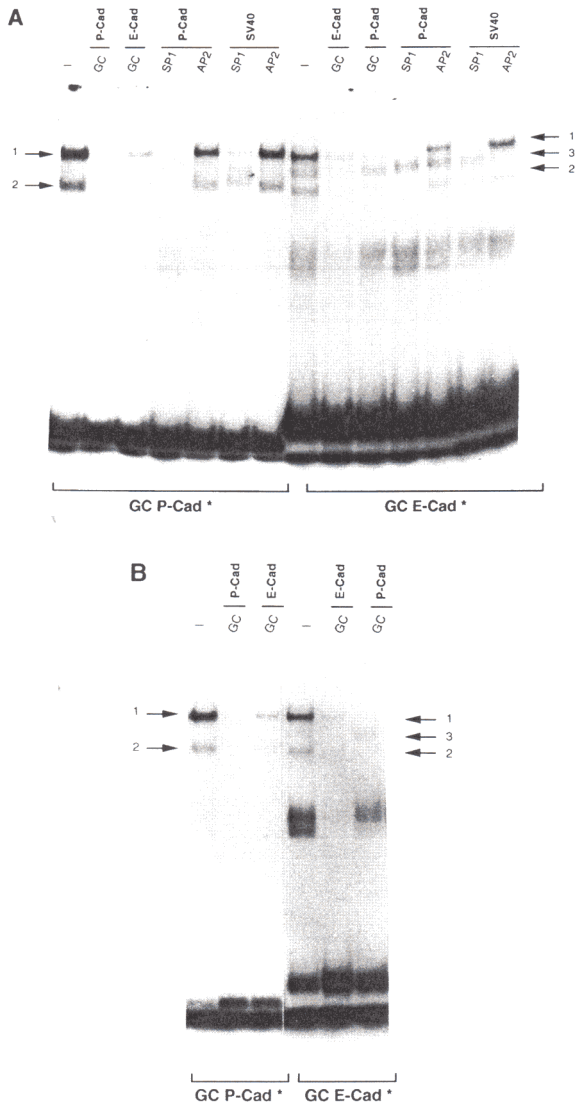

Figure 5. Band-shift analysis of the GC-rich regions of E- and P-cadherin promoters with HaCa4 and CarB extracts. Gel retardation assays and competition with the indicated oligonucleotides were performed with 2.5 μg of HaCa4 (A) and CarB (B) nuclear extracts as described in Figure 4B. The main retarded complexes observed with the GC.E probe are indicated by arrows on the right (complexes 1, 2, and 3), and those obtained with the GC.P probe are indicated on the left (complexes 1 and 2).

tivity of the –75 P-cadherin promoter in the different cell lines (about twofold to fourfold) giving rise to luciferase values close to those of the –200 construct in the different cell lines (50–80% activity). These results indicated that the GC-rich region of E-cadherin promoter was partially able to substitute for the homologous GC-rich region of the P-cadherin promoter.

The possible influence of the E-pal element of E-cadherin promoter on the activity of the heterologous P-cadherin promoter in murine keratinocytes was also analyzed by using a hybrid construct in which the E-pal element was inserted 5′ of the –200 P-cadherin promoter. A moderate inhibitory effect (40% inhibition) was observed in the activity of the –200 promoter in HaCa4 cells, whereas a much weaker effect or no significant effect was observed in the other three cell lines (data not shown).

DISCUSSION

The correct expression of functional cadherins is a crucial process in animal morphogenesis that needs precise spatio-temporal regulation. Alterations in cadherin expression occur in neoplastic processes and lead to the loss of cell-cell interactions, thus constituting an important mechanism of tumor progression and invasiveness [reviewed in 2]. To understand the molecular mechanisms involved in down-regulation of E- and P-cadherin expression in mouse skin carcinogenesis, we analyzed the promoter activities of both genes in a collection of murine keratinocytes representing different stages of mouse skin tumor progression. Several regulatory elements present in the 5′ promoter of the mouse and human E-cadherin gene were previously described and analyzed in other cell systems, and the results support a major positive role for a proximal GC-rich region and a CCAAT box and a repressor role for the palindromic E-pal element [22,23,25]. The regulatory elements of the P-cadherin promoter are still not well defined [28]. Based on sequence analysis, we previously postulated the existence of potential regulatory elements in the 5′ proximal region of the mouse P-cadherin promoter [29]. The transient transfection analysis reported here with different constructions of the 5′ upstream region of the P-cadherin promoter showed that positive elements leading P-cadherin transcription in expressing keratinocytes were concentrated in the nt –200 to +47 proximal region and suggested a regulatory role for the GC-rich (nt –101 to –80) and the CCAAT box (nt –65). Interestingly, the –200 P-cadherin promoter exhibited cell-type specificity and was basically inactive in non-expressing spindle cells. The role of the GC-rich and CCAAT regions in the transcriptional regulation of P-cadherin expression was further supported by the DNAse I footprinting analysis of the nt –200 to +120 region, where binding of nuclear factors was exclusively detected between nt –94 and –60, which includes most of the GC-rich region and the CCAAT box (Figure 3A and B). Specific retarded complexes were also detected with both regions in band-shift assays of nuclear extracts from expressing and non-expressing cells. Direct involvement of Sp1 factors in the recognition of the GC-rich region was established by supershift analysis (Figure 6). The nature of the factors binding to the P-cadherin CCAAT box remains unclear. Although the results of the gel retardation assays suggest the involvement of a CP1-related factor (Figure 4), an oligonucleotide containing a CP1 binding site could not prevent the CCAAT box protection detected in the footprinting

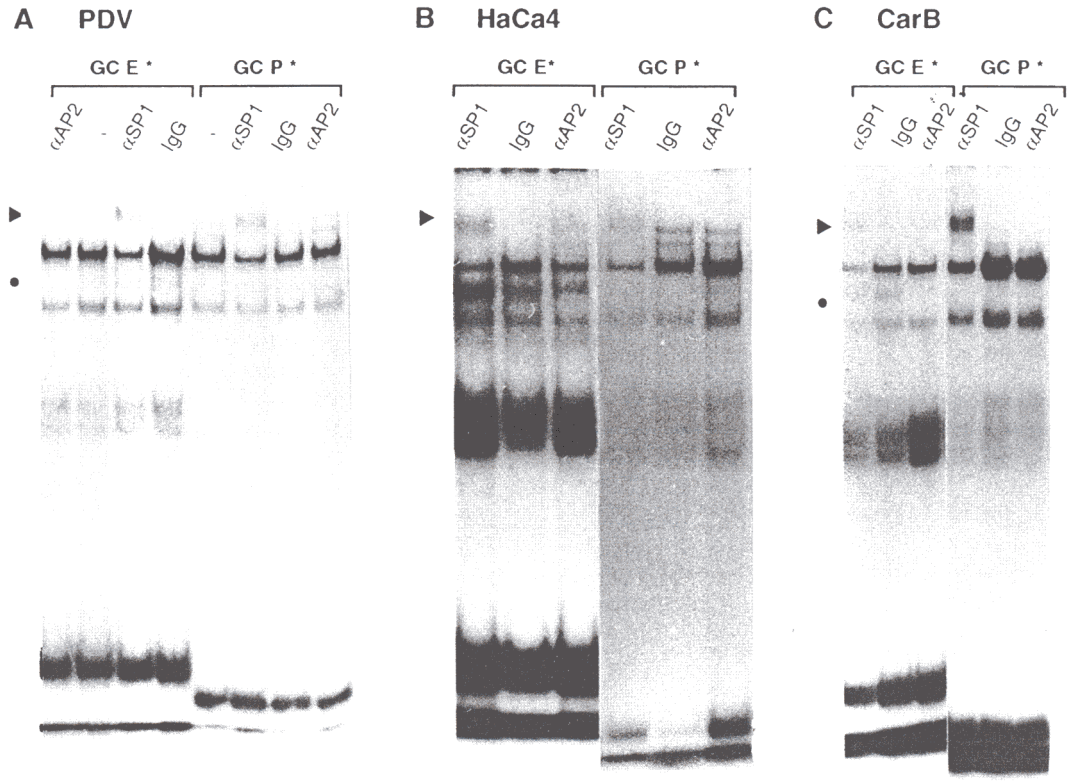

Figure 6. Supershift analysis of the GC-rich complexes of E- and P-cadherin promoters with nuclear extracts from PDV (A), HaCa4 (B), and CarB (C) cells. Gel retardation assays were performed with the GC.P and GC.E labeled probes in the absence (–) or presence of anti-Sp1 antibody, anti-AP2 antibody, or purified rabbit immunoglobulin G (1 μg/reaction) with nuclear extracts of the indicated cell lines. Supershifted complexes detected with anti-Sp1 and anti-AP2 antibodies are indicated by arrowheads, and the decreased complex 3 of the GC.E probe detected with anti-AP2 antibodies is indicated by a dot. The retarded complexes obtained in the absence and presence of immunoglobulin G in HaCa4 and CarB extracts were exactly the same.

analysis (data not shown). These partially discrepant results could be due to stabilization of the binding of CP1 (or a related factor) with the CCAAT box by protein-protein interactions with the adjacent Sp1 factor, as has been previously described for C/EBP-SP1 interactions in the rat cytochrome P4502D5 promoter [44]. In this context, it is interesting to note that the SP1.SV40 consensus oligonucleotide partially prevented the protection of the P-cadherin CCAAT box region in the footprinting reaction with PDV nuclear extracts (Figure 3B), suggesting that Sp1 factors could mediate the interaction of the CCAAT box with its cognate nuclear factor or factors. On the other hand, although the transient transfection assays with the ΔAP2 construct suggested that nt +14 to +47 is required for the optimal activity of the P-cadherin promoter (Figure 1B), direct interaction of the putative 3′ AP2 binding site (nt +30) with nuclear factors was not detected either in the footprinting analysis (Figure 3) or in gel retardation assays (data not shown). Alternatively, the decreased activity of the ΔAP2 construct can be explained by reduced stability or translational activity of the mRNA because of the deleted 5′ noncoding leader region (nt +14 to +47), as has been suggested for other cases such as the α- and β-globin [45] and yeast CAT mRNAs [46]. This possibility will be tested in future studies.

In the non-expressing spindle CarB cells, the different P-cadherin promoter constructs had signifi-

Table 3. Effect of the GC-Rich Region of the E-Cadherin Promoter on the Basal Activity of the P-Cadherin Promoter

	Activity of promoter constructs (% CMV-Luc)*			
Cell line	–200	–75	GC.E/–75	Fold-increase†
MCA3D	1.75	0.35	0.83	2.37
PDV	5.9	2.0	3.7	1.85
HaCa4	1.9	0.22	0.8	3.63
CarB	0.63	0.13	0.5	3.84

*The P-cadherin promoter constructs –200 and –75 are shown in Figure 1A. Construct GC.E/–75 was generated by inserting the GC-rich region of the E-cadherin promoter (nt –60 to –23) 5′ of the –75 construct as indicated in Materials and Methods. The activity of the constructs in the various cell lines was estimated as described in Materials and Methods and is given as the percentage of the activity driven by the CMV-Luc promoter. The data are averages obtained from two independent experiments, performed in duplicate.

†The fold-increase was derived from the ratio of the activities of the GC.E–75 and the –75 constructs.

cantly less activity than in the expressing keratinocyte cell lines. This low activity of the exogenous promoter could not be explained, however, by the results of binding studies of nuclear factors in the CCAAT box and GC-rich region, because similar results were obtained with PDV, HaCa4, and CarB nuclear extracts. Specifically, Sp1 factors seem to participate in the recognition of the GC-rich region in expressing and non-expressing cells. One possible explanation for the lack of activity of the exogenous P-cadherin promoter in CarB cells is the existence of different isoforms or differential modification of Sp1 factors or lack of interaction with specific coactivators rendering them inactive in CarB cells. This observation is also in line with a report on the regulation of keratin K18 gene expression in colon carcinoma cells, in which the binding of Sp1 factor differentially modulates the activity of the 5′ proximal promoter in two cell clones differing in the level of K18 expression by fivefold to sixfold [47].

The analysis of the E-cadherin promoter showed that the nt −178 to +92 construct exhibited cell-type specificity in the keratinocyte cell system (Figure 2 and Table 2), with positive involvement of the CCAAT box and GC-rich proximal region and a repressor role for the E-pal element, in agreement with previous observations in other cell systems [22,25]. The strongest repressor effect of the E-pal element, observed in spindle CarB cells devoid of promoter activity, strongly supports a dominant role for this negative regulator in undifferentiated cells. Interestingly, the footprinting analysis of the E-cadherin promoter detected distinct protection of the E-pal element and adjacent 5′ sequences in expressing and non-expressing keratinocytes (Figure 3C), supporting the existence of modifications in the factor or factors that recognize those sequences or in their interaction with coactivators between both cell types.

Comparison of the nuclear factors interacting with the CCAAT box and GC-rich region of the E- and P-cadherin promoters showed that although common nuclear factors appeared to participate in both promoters (CP1- and Sp1-related factors for the CCAAT box and GC regions, respectively), additional factors were involved in the recognition of both elements of the E-cadherin–promoter, giving rise to E-cadherin–specific nuclear complexes. CP2- and C/EBP-related nuclear factors seem to be responsible for the specific CCAAT complexes of the E-cadherin promoter, whereas AP2-related factors are mainly involved in the specific GC complexes of E-cadherin, as in other cell types [25]. Interestingly, the weaker protection of the proximal GC-rich region and the results of the band-shift assays support some kind of modification in the Sp1 or AP2 factors or their coactivators in E-cadherin-deficient HaCa4 and CarB cells. These alterations, together with the repressor effect of the E-pal element, could account for the low level or lack of activity of the E-cadherin promoter in those cell lines.

Taken together, the results obtained in this study suggest that the two promoters are regulated by different mechanisms with regard to the involved transcription factors, although the promoters appear very similar in their sequence composition (i.e., both lack TATA boxes and contain GC-rich regions and CCAAT boxes). As seen with other epithelial promoters, a significant degree of specificity seems to be derived from different combinations of general transcription factors rather than from the action of epithelium-specific factors [48–51]. This differential regulation of the two promoters in cultured keratinocytes can be related to the differential pattern of expression of both genes in the epidermis, P-cadherin being restricted to the basal proliferative layer and E-cadherin expressed in basal and suprabasal layers [7,11]. Our results also support the involvement of the E-pal element of E-cadherin promoter as an important repressor in non-expressing undifferentiated keratinocytes. On the other hand, modification or loss of some of the factors interacting with the regulatory regions of both promoters (such as in the Sp1- and AP2-related and E-pal–recognizing factors) seemed to occur in non-expressing malignant keratinocytes, which may therefore play a role in the down-regulation of E- and P-cadherin in mouse skin carcinogenesis. Further studies will be needed to clarify the interactions among the different factors and to definitively characterize the complex pattern of modulation of E- and P-cadherin gene expression. Nevertheless, our results contribute to the limited knowledge on the regulation of cadherin gene expression in normal epidermis and in skin carcinogenesis.

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