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"DINÁMICA DE TFIIH Y SU ENSAMBLAJE NUCLEAR DURANTE EL DESARROLLO TEMPRANO DEL EMBRIÓN DE *Drosophila melanogaster*"

TESIS

QUE PARA OBTENER EL GRADO DE DOCTOR EN CIENCIAS

PRESENTA

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DIRIGIDA POR

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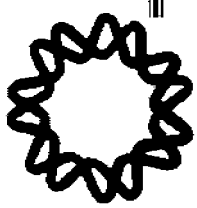

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ÍNDICE GENERAL.

PÁGINA.

ÍNDICE DE FIGURAS.

PÁGINA.

ABSTRACT.

We present the first analysis of the subcellular dynamics of the transcription DNA-repair factor TFIIH at the onset of transcription in early *Drosophila* development. TFIIH is composed of ten polypeptides that are part of two complexes – the core and the CAK. We found that the TFIIH core is initially located in the cytoplasm of syncytial blastoderm embryos, and that after mitotic division ten and until the cellular blastoderm stage, the core moves from the cytoplasm to the nucleus. By contrast, the CAK complex is mostly cytoplasmic during cellularization and during gastrulation. However, both components are positioned at promoters of genes that are activated at transcription onset. Later in development, the CAK complex becomes mostly nuclear and co-localizes in most chromosomal regions with the TFIIH core, but not in all sites, suggesting that the CAK complex could have a TFIIH-independent role in transcription of some loci. We also demonstrate that even though the CAK and the core coexist in the early embryo cytoplasm, they do not interact until they are in the nucleus and suggest that the complete assembly of the ten subunits of TFIIH occurs in the nucleus at the mid-blastula transition. In addition, we present evidence that suggests that DNA helicase subunits XPB and XPD are assembled in the core when they are transported into the nucleus and are required for the onset of transcription.

RESUMEN.

La transcripción no es activa durante las primeras etapas del desarrollo de organismos multicelulares. Hasta el momento no se sabe cúal o cuales son los mecanismos que activan el inicio de la transcripción cigótica. En el presente trabajo mostramos la dinámica subcelular y del ensamblaje del factor de reparación del DNA y transcripción TFIIH durante el inicio de la transcripción en el desarrollo temprano de *Drosophila*. TFIIH es un complejo de proteínas compuesto por 10 polipéptidos que son parte de dos subcomplejos: el "core" y el complejo de Cinasa activadora de Cdk (CAK). En este estudio, encontramos que el "core" de TFIIH está localizado inicialmente en el citoplasma de los embriones en blastodermo sincicial; mientras que a partir de la división mitótica 10 y hasta la etapa de blastodermo celular, el "core" se mueve del citoplasma hacia el interior del núcleo. En cambio, el subcomplejo CAK está preferencialmente en el citoplasma durante el blastodermo celular y la gastrulación. Sin embargo, ambos subcomplejos están posicionados sobre promotores de genes que se activan durante el inicio de la transcripción. Más tarde en el desarrollo de la mosca, en células de glándulas salivales de larvas del tercer estadio, el CAK es observado en el núcleo y co-localiza en muchas regiones cromosomales con el "core", pero no en todos los sitios, sugiriendo que el CAK puede tener una función independiente de TFIIH en la transcripción de algunos genes. También, demostramos que aún cuando el CAK y el "core" coexisten en el citoplasma del embrión temprano, ellos no interactúan hasta que ambos están dentro del núcleo, sugiriendo que el ensamblaje de las 10 subunidades de TFIIH ocurre en los núcleos durante el inicio de la activación de la transcripción. Finalmente, presentamos evidencias que sugieren que el ensamblaje de XPB y XPD en el "core" es importante tanto para su movimiento del citoplasma al núcleo así como para el inicio de la transcripción en *Drosophila*.

1. INTRODUCCIÓN.

1.1. El factor transcripcional IIH (TFIIH).

TFIIH es un complejo multiproteico que participa en procesos celulares esenciales como la reparación del DNA, el control del ciclo celular y la transcripción mediada por la RNA polimerasa I (RNA pol I) y II (RNA pol II). TFIIH está formado por un núcleo o "core", constituido por las subunidades XPD, XPB, p62, p52, p44 y p34 (Feaver et al., 1993). Recientemente se ha descubierto una nueva subunidad del "core" llamada TTDA/p8 (Figura 1A; Giglia-Mari et al., 2004; Ranish et al., 2004). TFIIH también está formado por un subcomplejo de cinasa activadora de cinasas dependientes de ciclinas ("cdk-activating kinase" o CAK), compuesto por las proteínas MAT1, ciclina H1 (CycH) y la cinasa dependiente de ciclina CDK7 (Flores et al., 1992; Gerard et al., 1991; LeRoy et al., 1998; Tirode et al., 1999). Las proteínas del "core" de TFIIH intervienen en la reparación del DNA, particularmente en la reparación por escisión de nucleótidos (REN). El CAK participa en el control del ciclo celular. Por otra parte, el "core" y el CAK participan, conjuntamente con la RNA polimerasa II, en el inicio de la transcripción (Roy et al., 1994a).

La estructura tridimensional del complejo TFIIH de humano (Schultz et al., 2000) y de levadura (Chang y Kornberg, 2000) muestran que TFIIH forma una estructura tipo anillo con una protuberancia. El anillo corresponde al núcleo, en el cual XPB y XPD se ubican una enfrente de la otra, sin interaccionar físicamente, mientras que la protuberancia corresponde al CAK (Figura 1B; Chang y Kornberg, 2000; Schultz et al., 2000). De las proteínas que componen a TFIIH, solamante XPB, XPD, p44 y CDK7 tienen actividad enzimática. Las dos primeras son helicasas del DNA, p44 posee la actividad de E3 ligasa de ubiquitina y la última es una cinasa dependiente de ciclina. Además, XPB, XPD y CDK7 son proteínas que presentan actividad de ATPasa (Roy et al., 1994b; Kornberg et al., 2005).

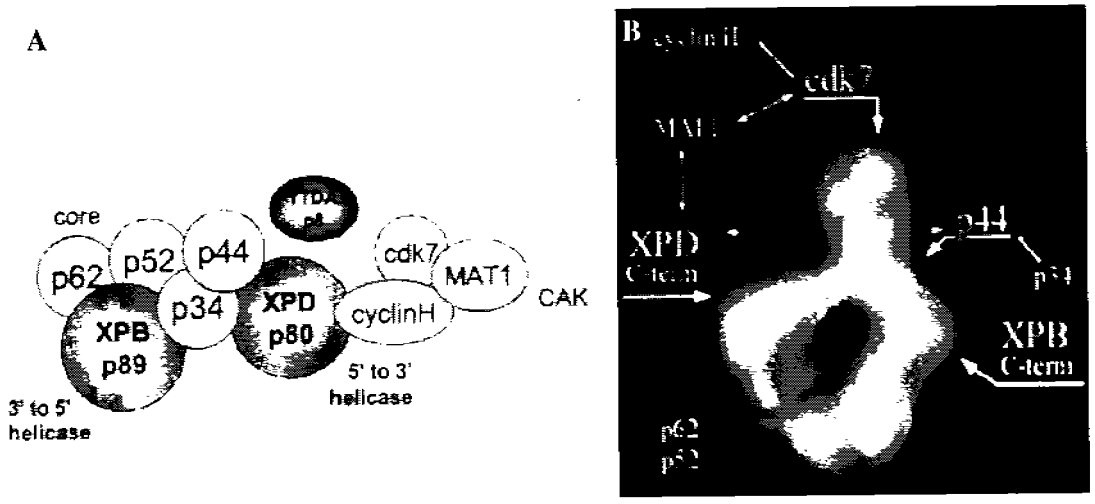

Figura 1. Componentes y estructura molecular de TFIIH. (A) Componentes de TFIIH. 7 subunidades componen el "core", p34, p44, p52, p62, p8, XPD y XPB. Cuando el CAK (cdk7, ciclina H y MAT1) se une al "core" por medio de XPD, TFIIH participa en la transcripción mediada por la RNA pol I y II. La subunidad número 10 de TFIIH, TTDA/p8, contribuye a la estabilidad y concentración de TFIIH en la célula, y en la protección de la degradación *in vitro*. Mutaciones en las proteínas en color gris producen Tricotiodistrofia, TTD (Cleaver, 2005). (B) Estructura molecular de TFIIH humano. En la figura se observa la organización de las subunidades de TFIIH. La posición de las subunidades cdk7, XPD, XPB y p44 son inferidas a partir de experimentos de inmunomarcaje. Las subunidades ciclina H, MAT1, p34, p62 y p52 son ubicadas de acuerdo a estudios de interacción proteína-proteína (Tomado de Schultz et al., 2000).

1.2. TFIIH en la reparación por escisión de nucleótidos.

La reparación por escisión de nucleótidos (REN) remueve regiones que presentan distorsiones en la molécula del DNA, como las generadas por irradiación con luz ultravioleta (UV). La luz ultravioleta genera dos tipos de lesiones en el DNA, los dímeros de ciclobutano de pirimidinas (dímeros de timina) y el fotoproducto 6-4 (6-4 PP). El "core" de TFIIH participa removiendo las lesiones causadas por la irradiación con luz ultravioleta. Existen dos tipos de REN, la reparación general del genoma (REN-GG) y la reparación acoplada a la transcripción (REN-AT). La REN-GG se encarga de remover los dímeros de timina de todo el genoma y es un proceso lento. En cambio la REN-AT, es un proceso que consiste en remover rápidamente el daño de las regiones que se transcriben. Ambos

mecanismos de reparación difieren en la forma de reconocer la distorsión en el DNA (De Laat et al., 1999). De manera general, el mecanismo de REN se lleva acabo en dos etapas: primero, hay una doble incisión ("nick") en la hebra del DNA dañado por dos endonucleasas, cada una actúa sobre los extremos de la base dañada; segundo, se sintetiza el fragmento escindido.

En la REN-GG, el daño es reconocido por la proteína UV-DDB, la cual se une a la lesión 6-4PP. En seguida, UV-DDB permite al complejo heterodimérico, conformado por las proteínas XPC y HHR23B, unirse a la lesión (Moser et al., 2005; Evans et al., 1997). Una vez que se detectó el daño por XPC-HHR23B, este complejo recluta al "core" de TFIIH (Figura 2; Lyer et al., 1996). Este último abre el DNA mediante la acción de XPB (3'-5') y XPD (5'-3'). Estudios con DNA dañado y pre-desnaturalizado, revelan que la separación de las hebras del DNA no es la única función de TFIIH, sino que se requiere para una mayor eficiencia en los pasos siguientes (Mu y Sancar, 1997). Se tiene evidencia que durante la REN, TTDA se une a TFIIH para estabilizarlo (Figura 3; Giglia-Mari et al., 2006). Después se unen las proteínas RPA y XPA las cuales son factores que estabilizan al complejo. En la ausencia de la proteína XPA no se forma un complejo estable de pre-iniciación de la REN y consecuentemente no se realiza esta última (Mu et al., 1997; Evans et al., 1997). La proteína RPA estimula la interacción entre la proteína XPA y el DNA resultando la formación del complejo RPA-XPA en el sitio dañado (Li et al., 1995; Stigger et al., 1998; Lee et al., 2003). Sin embargo, a diferencia de la proteína XPA, la proteína RPA tiene otras funciones en el metabolismo del DNA, como en la replicación de este último, recombinación y en el ciclo celular (He et al., 1995; Jones y Wood, 1995; Buns et al., 1996; Köberle et al., 2006). Posteriormente, se reclutan las endonucleasas de cadena sencilla ERCC1-XPF y XPG que cortan los extremos del fragmento dañado. Finalmente, la DNA polimerasa ε sintetiza el fragmento escindido utilizando la cadena intacta como molde (De Laat et al., 1999). También, la DNA polimerasa ε participa en el mecanismo de reparación por escisión de bases y en la recombinación en respuesta al DNA dañado (Wang et al., 1993; Jessberger et al., 1993; Jessberger et al., 1996). Una vez reparado el daño en las regiones transcripcionalmente activas es posible reiniciar la transcripción.

Por otro lado, la reparación tipo REN-AT inicia cuando la RNA polimerasa II se detiene en un sitio donde existe un daño en la hebra del DNA templado. Posteriormente, esta

anormalidad es detectada por las proteínas CSA-CSB (Henning et al., 1995; You et al., 1998), continuando con un mecanismo similar al REN-GG.

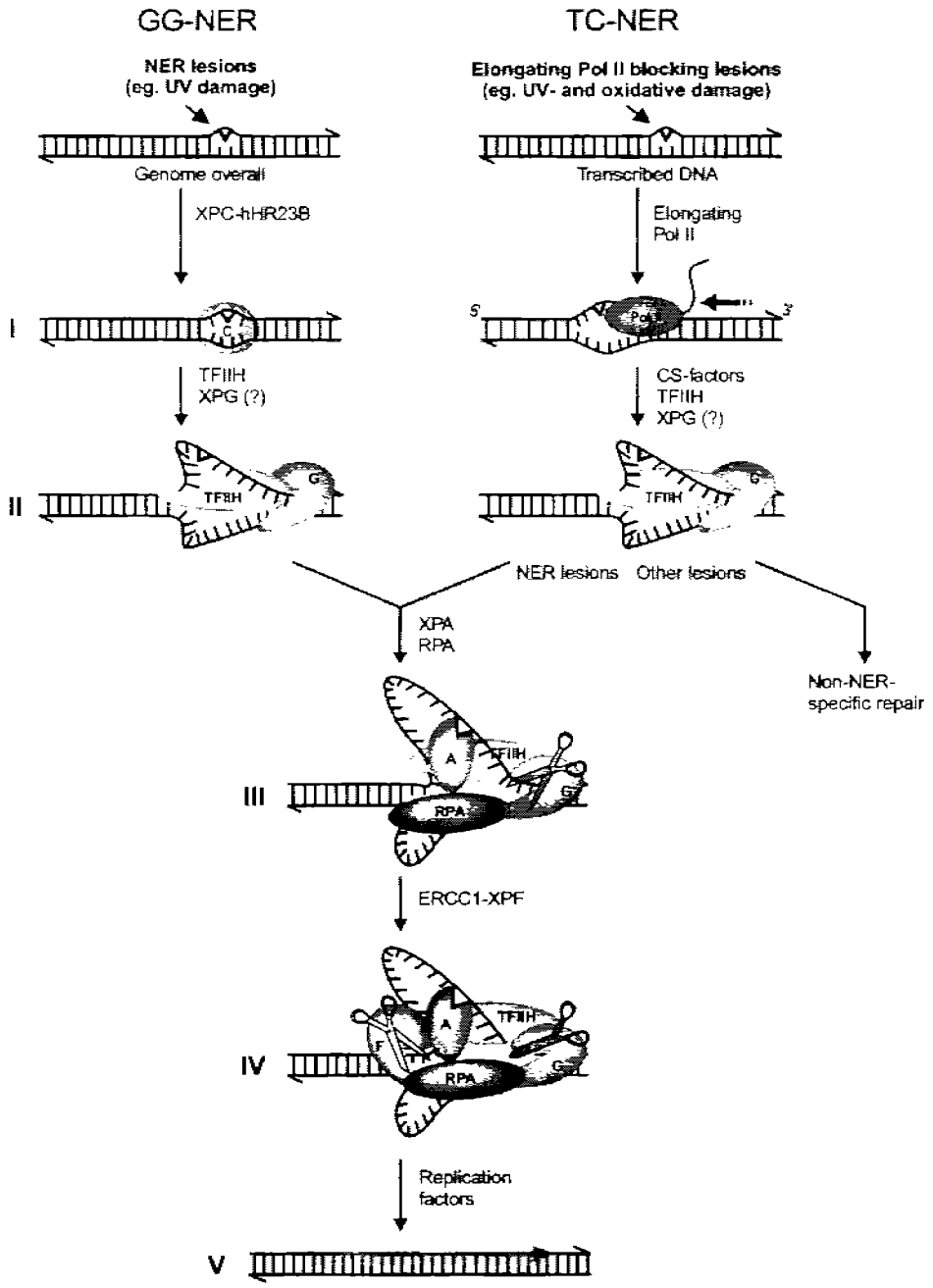

Figura 2. Modelo molecular para reparación por escisión de nucleótidos (Tomado de De Laat et al., 1999).

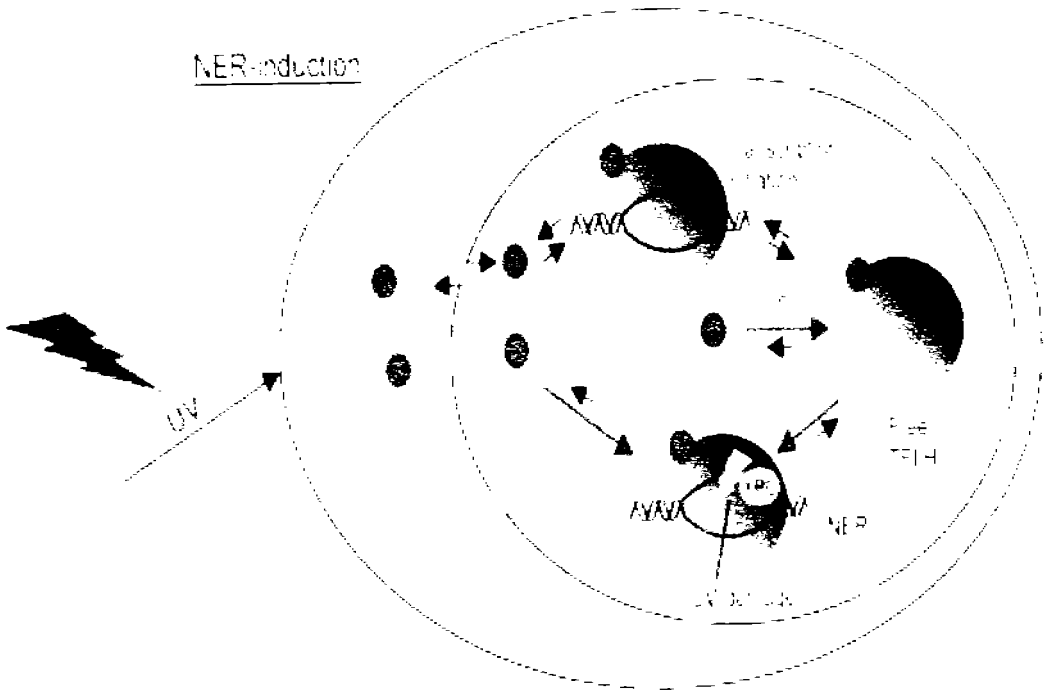

Figura 3. TTDA/p8 tiene dos cinéticas diferentes en células viva expuestas a luz ultravioleta. Una de ellas muestra a TTDA unida a TFIIH y la otra muestra a una proteína TTDA libre en el citoplasma y núcleo. Durante REN, TTDA se une más a TFIIH para estabilizarlo. TTDA en verde y TFIIH en anaranjado (Tomado de Giglia-Mari et al., 2006).

1.3. TFIIH participa en la transcripción mediada por la RNA polimerasa I y II.

El DNA es una macromolécula que funciona como reservorio de la información genética. El proceso celular que se encarga de transmitir esta información mediante la síntesis de un RNA mensajero (mRNA) se denomina transcripción. La enzima responsable de la síntesis de los mRNA en eucariotes es la RNA pol II. La transcripción mediada por la RNA pol II se divide en tres fases: la iniciación, la elongación y la terminación. La iniciación consiste en el reconocimiento de la región promotora y la formación del complejo de iniciación, el cual se compone de factores multiproteicos denominados TFIIx y la RNA pol II. En algunas secuencias, el primer paso de la iniciación consiste en el ensamblaje de la maquinaria basal de transcripción. Primero, el promotor es reconocido por el complejo TFIID, el cual está compuesto por la proteína de unión a la caja TATA (TBP) y sus proteínas asociadas (TAFs). Posteriormente, se recluta TFIIA, al parecer para incrementar

la afinidad de la TBP por la caja TATA. Después TFIIF se une a la RNA pol II permitiendo la unión de la polimerasa al complejo de iniciación (Dvir et al., 2001). Finalmente TFIIE se une al complejo de iniciación y recluta a TFIIH (Dvir et al., 2001; Spangler et al., 2001) mediante la interacción directa con la proteína XPB (Maxon et al., 1994). Con todo lo anterior queda establecida la maquinaria basal de transcripción. El siguiente paso de la iniciación de la transcripción requiere de la separación de la doble hebra del DNA (formación del complejo abierto). La separación del DNA requiere de la presencia de enzimas con actividad de helicasa para el DNA, dicha actividad se encuentra en el complejo TFIIH, y en particular en las helicasas XPB y XPD (Coin et al., 1999; Tirode et al., 1999). Una vez separadas las hebras del DNA, se inicia la síntesis del mRNA. El paso inicial de la síntesis es la formación del primer enlace fosfodiester. Estudios *in vitro* han demostrado que la actividad de XPB es esencial para la formación de dicho enlace (Bradsher et al., 2000). Posteriormente, se inicia la elongación, la cual da comienzo cuando el carboxilo terminal de la subunidad grande de la RNA pol II (CTD) es fosforilado. Esta fosforilación se requiere para que la polimerasa se libere de los TFIIx. Una de las cinasas responsables de la fosforilación del CTD es CDK7, la cinasa de TFIIH (Dahmus, 1995). La capacidad de fosforilar al CTD por parte de CDK7 está a su vez regulada por fosforilación (Larochelle et al., 2001). Durante la primera fase de la elongación, la polimerasa no puede "escapar" del promotor, y sintetiza fragmentos cortos de mRNA, lo que se denomina transcripción abortiva (Dvir et al., 2001). Mediante la acción de la helicasa XPB, la RNA pol II finalmente escapa del promotor y se inicia la elongación propiamente dicha (Figura 4). La importancia de XPB en este proceso se estableció mediante el uso de mutantes en el dominio de ATPasa. Se observó que la pérdida de la actividad de XPB impide la elongación (Bradsher et al., 2000; Moreland et al., 1999). La terminación es un proceso menos claro, TFIIH no participa en la terminación. Se ha reportado que la señal de poliadenilación del pre-mRNA funciona como una secuencia necesaria para la terminación *in vivo*. Experimentos en levadura sugieren que algunos elementos que componen la señal de poliadenilación provocan la detención y el retrazo de la polimerasa durante la transcripción, haciendo que se liberen los factores de elongación, permitiendo el procesamiento del pre-mRNA y la terminación (Connelly et al., 1998; Orozco et al., 2002; Kim et al., 2004; Nag et al., 2006).

Recientemente, se demostró que existe una serie de proteínas que son necesarias para establecer contacto entre los reguladores transcripcionales y la maquinaria basal. Estas proteínas se han agrupado en el complejo denominado mediador. Inicialmente, el mediador fue aislado en levaduras. Este complejo está formado por al menos 20 proteínas y tiene la capacidad de estimular la transcripción *in vitro* (Kim et al., 1994). Posteriormente se demostró la interacción entre este mediador y TFIIH, en particular con Kin28, el homólogo de CDK7 en levadura. Dicha interacción es indispensable para que se lleve acabo la transcripción *in vitro* (Sakurai y Fukasawa, 2000). A diferencia de lo observado en levadura, la relación del mediador y TFIIH en organismos superiores es mucho más compleja. Se ha demostrado la existencia de varios complejos tipo mediador, los cuales tienen funciones específicas en la regulación transcripcional, ya sea negativa o positivamente. En particular, se ha demostrado que el mediador contiene a CDK8, y esta proteína a su vez regula negativamente la actividad de cinasa de TFIIH. La inactivación de CDK7 se lleva acabo de manera indirecta mediante la fosforilación de Ciclina H1 por CDK8. Este tipo de regulación en la transcripción, realizada mediante la modificación de la actividad de dos Cdks, únicamente se ha podido caracterizar en células de mamífero (Akoulitchev et al., 2000).

Varias publicaciones recientes reportan que hay evidencia de la participación de TFIIH con la RNA pol I en la transcripción de los RNA ribosomales. Se observó que TFIIH tiene una distribución homogénea en el núcleo, pero además una acumulación nucleolar (Vermeulen et al., 2002; Egly et al., 2002). En sistema realizado para evaluar el inicio de la transcripción mediada por la RNA pol I, que carece de TFIIH, da como resultado un sistema transcriptionalmente inactivo. Además, mutantes en levadura de CDK7 y p62 muestran un decremento en la síntesis de pre-rRNA 35S. Experimentos *in vitro* muestran que las actividades de ATPasa, helicasa y de cinasa de TFIIH no son requeridas para la activación de la transcripción por la RNA pol I, pero si se requieren para el escape del promotor, elongación y reiniciación (Iben et al., 2002).

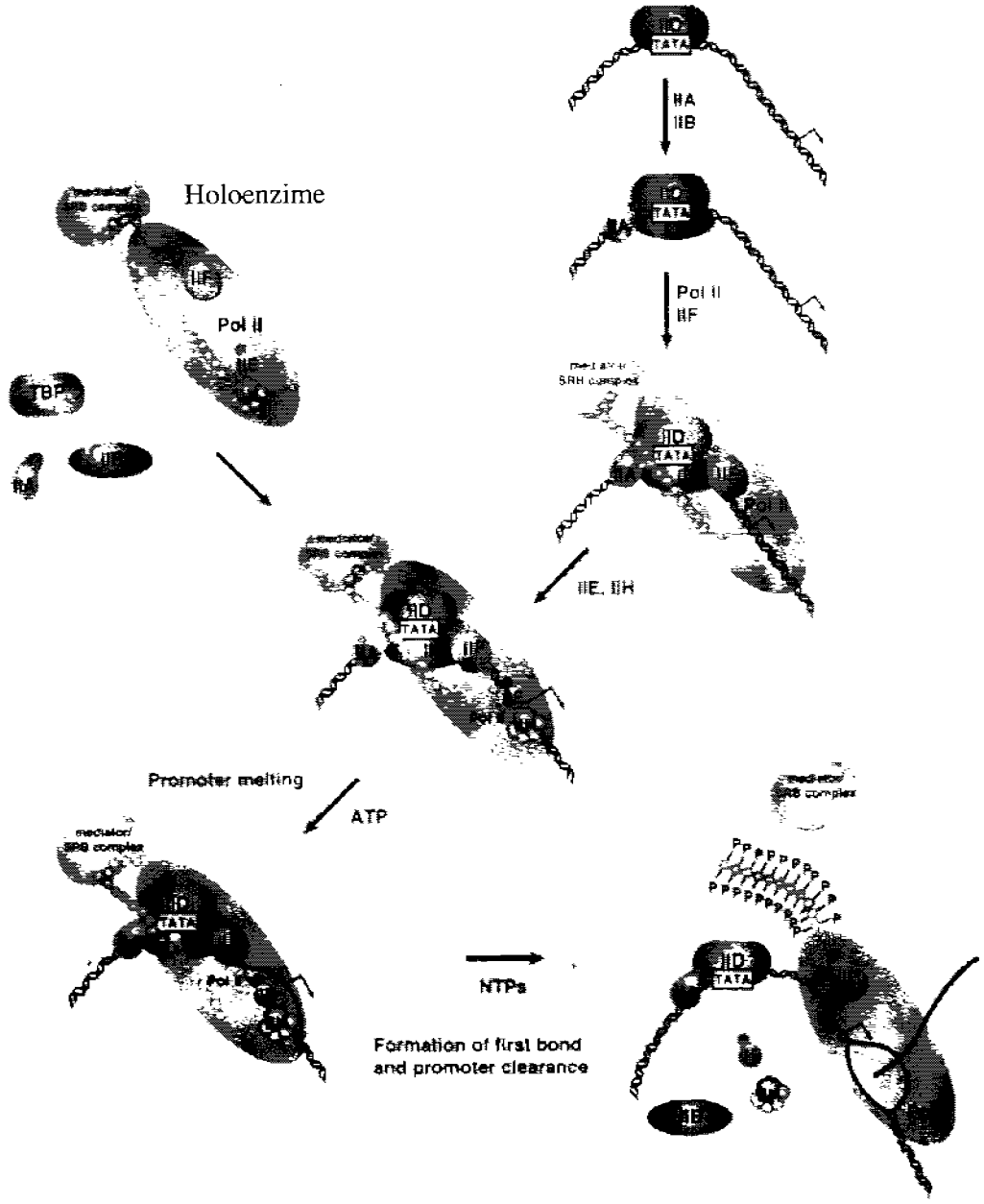

Figura 4. Modelo del inicio de la transcripción mediado por la RNA polimerasa II. El modelo de la holoenzima representa la abreviación del modelo por pasos, en el cual un subgrupo ensamblado de factores generales de la transcripción (GTFs) y la RNA pol II se unen en un paso al promotor (Tomado de Orphanides et al., 1996).

1.4. TFIIH participa en la regulación del ciclo celular.

La participación del CAK de TFIIH en la regulación de ciclo celular aún no es muy clara (Figura 5). Estudios en algunas mutantes de *Drosophila*, han podido relacionar la capacidad de fosforilación del complejo CAK con la activación de los complejos cdc2/ciclina A y cdc2/ciclina B. Estos datos correlacionan con el fenotipo de detención del ciclo celular que se presenta al mutar a *Dmcdk7*, el cual es idéntico al que presentan las mutantes en cdc2 (Suter et al., 1998). Es importante mencionar que algunas mutantes no presentan problemas en la progresión del ciclo celular, pero si en la transcripción (Leclerc et al., 2000).

Por otra parte, se sabe que la helicasa XPD regula negativamente la función del CAK en el ciclo celular. La sobre-expresión de XPD induce una disminución en la actividad de CAK, resultando una baja en la fosforilación del T-loop (dominio característico de las cinasas dependientes de ciclinas) de Cdk1, defectos mitóticos y de letalidad. Por el contrario, la disminución en la expresión de XPD incrementa la actividad del CAK y de proliferación celular. En otras palabras, XPD es regulado en el inicio de la mitosis, cuando Cdk1, un blanco de CDK7 en el ciclo celular, es más activo. La regulación de XPD contribuye a la modulación de la actividad mitótica del CAK y, así mismo, a la progresión mitótica (Suter, et al., 2003).

Se sabe que cuando una mutante negativa de CDK7 se sobre-expresa en el embrión de la mosca, se observa un defecto en la expresión del transcrito del gen *fushi tarazu* (*ftz*), el cual es uno de los genes cigóticos que se transcribe a partir del blastodermo sincicial (ver adelante). Además, esta anormalidad se acompaña de una división nuclear extra en el dominio de la expresión de la mutante negativa. Previamente había sido descrito que la terminación del programa materno de la división nuclear en los embriones de *Drosophila* está bajo el control de la transcripción cigótica. Pero al sobre-expresar la mutante negativa de CDK7, la coordinación o el programa de división del ciclo celular y precisamente la división nuclear trece no presenta modificación o retraso. Tampoco hay diferencias en el nivel de fosforilación de cdc2. Estos datos sugieren posiblemente que otra cinasa es afectada por la expresión de la mutante negativa de CDK7 y que esta cinasa podría satisfacer la función de la regulación del ciclo celular en la ausencia de una proteína CDK7 normal en los embriones. Otra interpretación es que los niveles de fosforilación de cdc2 permanecen bajos durante el control del ciclo celular, ya que no han podido ser detectados

por las técnicas empleadas hasta ahora. Al parecer el defecto en el ciclo celular retrasó la activación de la transcripción cigótica más que afectar propiamente dicho el ciclo celular, ya que se observó a la RNA pol II transcripcionalmente activa después de la formación de las células polares o germinales. La formación de las células polares ocurre entre los ciclos nucleares nueve y once (Leclerc et al., 2000).

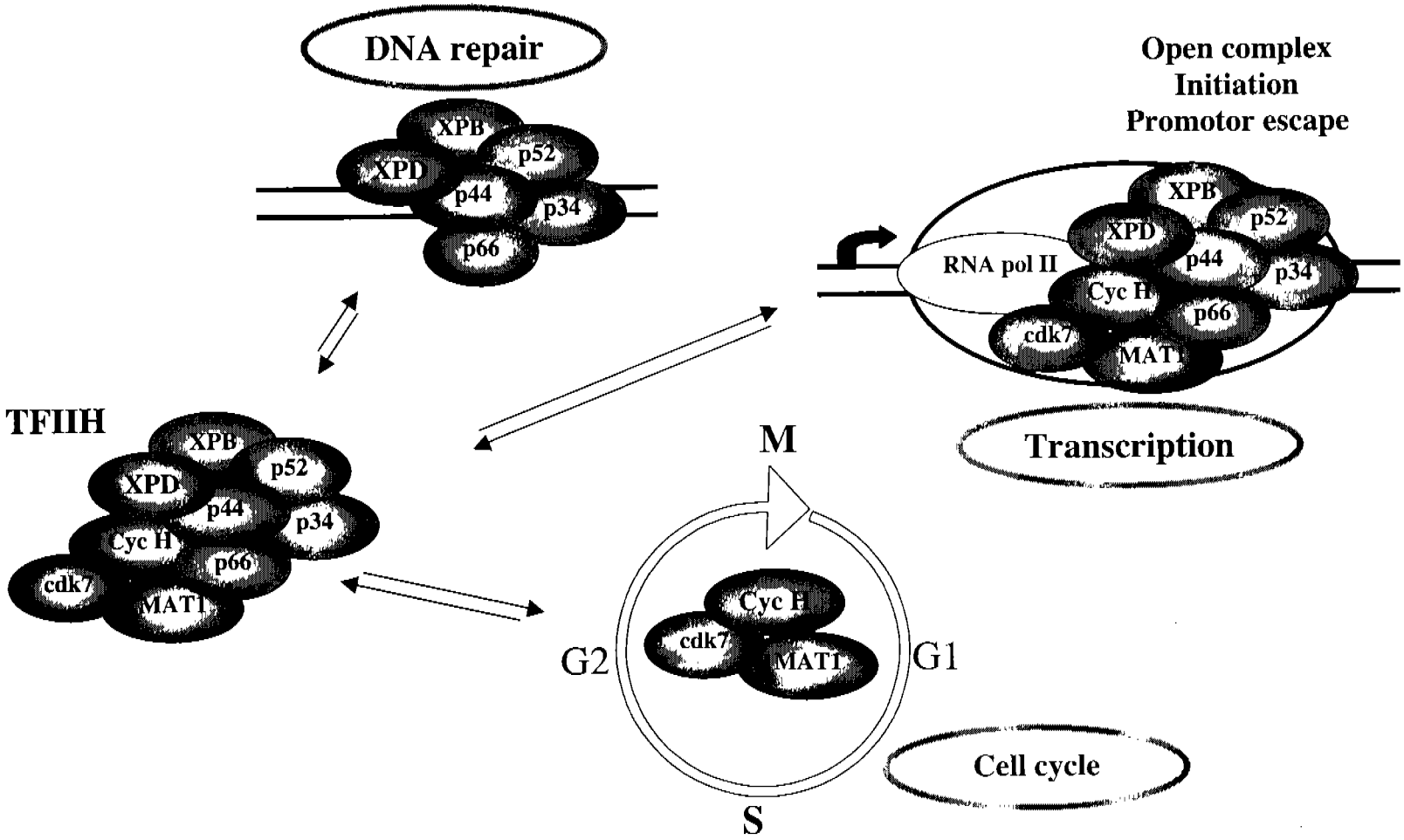

Figura 5. El papel de TFIIH en la transcripción, reparación del DNA y ciclo celular. Esquema modificado de Egly, 2001.

1.5. Otras cinasas dependientes de ciclinas (Cdks) fosforilan al dominio C-terminal (CTD) de la RNA polimerasa II.

Las tres cinasas dependientes de ciclina CDK7, CDK8 y CDK9, tienen una conexión con la maquinaria de transcripción y son reguladas por una expresión constitutiva de ciclinas. Estas Cdks pueden fosforilar el CTD de la subunidad grande de la RNA polimerasa II (cinasas CTD; Dahmus, 1994). Existen dos estados de fosforilación del CTD: la hipofosforilación y la hiperfosforilación. La hipofosforilación del CTD es una característica del complejo de preiniciación. La hiperfosforilación del CTD correlaciona con el inicio de la elongación de la transcripción (Dahmus, 1996). El CTD consiste en una serie de heptámeros repetidos (YSPTSPS), cinco aminoácidos de cada uno de los heptámeros pueden ser fosforilados por las cinasas antes mencionadas. El número de los heptámeros incrementa con la complejidad del organismo: 26 en levadura, 32 en *C. elegans*, 45 en *Drosophila* y 52 en mamíferos.

Las funciones de CDK7, CDK8 y CDK9 están relacionadas con las diferentes etapas de la transcripción (Roy et al., 1994a). Por ejemplo, CDK7 es una cinasa que fosforila al CTD después de la formación del complejo de preiniciación. La comparación entre la actividad de cinasa del CAK y TFIIH revela diferencias en la especificidad del sustrato: el CAK sin el "core" de TFIIH, muestra una fuerte preferencia por fosforilar a CDK2, la cual tiene una función en el ciclo celular, pero no puede fosforilar a la RNA pol II; TFIIH ("core" y CAK) fosforila a la RNA pol II, pero en esta situación CDK2 no es un sustrato para el CAK (Rossignol, 1997; Yankulov y Bentley, 1997).

Por otra parte, antes de que inicie el ciclo de transcripción, la holoenzima, la cual es un complejo que incluye a la RNA pol II que puede transcribir a partir de un promotor con un mínimo de factores generales, puede encontrarse asociada con CDK8. Esta cinasa puede producir solamente el estado de hipofosforilación del CTD de la RNA pol II. CDK8 preferentemente fosforila a la serina 5 de los heptámeros del CTD. De esta manera CDK8 podría prevenir que la holoenzima pase a ser un complejo de preiniciación de la transcripción. Todavía la función de CDK8 no es clara, lo que se hipotetiza es que actúa como un regulador negativo del complejo de preiniciación de la transcripción (Hengartner et al., 1998; Liao et al., 1995). También se sabe que CDK8 fosforila a la ciclina H1 del

subcomplejo CAK, esta fosforilación reprime la función de cinasa de CDK7, la cual es necesaria para el inicio la transcripción (Akoulitchev, 2000).

El potencial de elongación de la RNA pol II es controlado negativa y positivamente por los factores transcripcionales de la elongación. Un factor positivo de la elongación es CDK9. Esta cinasa principalmente fosforila a la serina 2 de los heptámetos del CTD de la RNA pol II en una etapa temprana de la elongación, haciendo que la RNA pol II entre a una etapa de elongación activa (Marshall et al., 1996; Price, 2000).

1.6. Modelo de fosforilación del CTD durante la transcripción.

Experimentos *in vivo* han demostrado que la RNA pol II es fosforilada en la serina 2 y serina 5 del heptámero del CTD. El estado de fosforilación de los heptámeros durante la transcripción pueden afectar la fosforilación del CTD dada por las diferentes Cdks asociadas a la transcripción. En el modelo de la fosforilación del CTD durante la transcripción se propone que después del escape del promotor (Krumm, 1995; Price, 2000), debido a la fosforilación del CTD por CDK7, existe una transcripción pausada durante la cual el CTD de la RNA pol II se encuentra desfosforilado en la serina 2. Inmediatamente después, CDK9 fosforila a la serina 2, dando paso a la elongación y al "capping" (Cho, 1999). El "capping" del mRNA ocurre inmediatamente después del inicio de la transcripción (Ho et al., 1998). En esta etapa de maduración del mRNA, el 5´ del primer nucleótido naciente es removido por la RNA 5´-trifosfatasa. Después, una guanililtransferasa dona un GMP, el cual es derivado de un GTP. La guanililtransferasa es la enzima que interacciona físicamente con el CTD fosforilado (Ho y Shuman, 1999). Finalmente, la metiltransferasa adiciona un grupo metilo al motivo GMP, completando una estructrura típica del "Cap" (Proudfoot et al., 2002). Subsecuentemente durante la elongación, la fosfatasa Ssu72 desfosforila a la serina 5 del CTD permitiendo así una correcta y eficiente terminación del transcrito (Krishnamurthy et al., 2004).

1.7. Importancia médica de TFIIH.

La información genética contenida en el DNA requiere de un mantenimiento que debe ser realizado de generación en generación. El DNA es sensible a daños causados por factores químicos y físicos durante la replicación, transcripción y recombinación. Estos factores

pueden causar diferentes tipos de lesiones. Por ejemplo, la luz UV genera dímeros de timina, los cuales son removidos principalmente por el mecanismo de reparación por escisión de nucleótidos. Este mecanismo genera una cadena de DNA sin lesión al remover la cadena dañada (Friedberg et al., 1992). Uno de los componentes importantes en el sistema de REN en eucariotes es TFIIH.

Existen varias enfermedades genéticas conocidas que pueden ser causadas por mutaciones en genes que participan en la reparación del DNA y/o en la transcripción. Entre los genes que causan estas enfermedades se encuentran algunos de los componentes de TFIIH. Ejemplos de estas enfermedades son: Xeroderma pigmentosum (XP), síndrome de Cockayne (SC) y Tricotiodistrofia (TTD). XP se ha asociado a una reparación deficiente del DNA, afectando particularmente a la reparación por escisión de nucleótidos. Pacientes afectados por XP tienen sensibilidad a la luz solar y pueden desarrollar cáncer (Seroz et al., 1995; Vermeulen et al., 1994). El SC se ha atribuido a un defecto en la reparación acoplada a la transcripción y a una recuperación lenta de la transcripción después de la reparación del DNA (Lehmann, 1995). La TTD se ha relacionado con defectos transcripcionales, como son la deficiencia transcripcional de genes que codifican para algunas queratinas (Vermeulen et al., 2000; Bergmann y Egly, 2001). Las dos últimas enfermedades también presentan sensibilidad a la luz solar, pero no desarrollan cáncer. Así también, la TTD y el SC se caracterizan por tener defectos en el desarrollo del sistema nervioso. Por ejemplo, los humanos con TTD y SC tienen retraso mental, pero además, los pacientes con SC presentan microcefalia, degeneración y disfunción neuronal. Esta última característica clínica es causada por la desmielinización de los axones (Itin y Pittelkow 1990; Nance y Berry, 1992).

Por otra parte, se sabe que pacientes con XP presentan características clínicas similares a pacientes con SC, esta última enfermedad es causada por mutaciones en las proteínas CSA y CSB. Estas características clínicas son comunes debido a que estas proteínas están involucradas en el proceso de la reparación acoplada a la transcripción de los daños causado por la luz UV. Otro argumento del por qué XP y SC presentan características clínicas en común, es que la ausencia de CSB y mutaciones en XPD y XPB, las cuales conducen al SC, debilitan la interacción entre la RNA polimerasa I y TFIIH (Vermeulen et al., 2002; Bradsher et al., 2002).

1.8. Importancia de la Biología y la Genética del Desarrollo de *Drosophila.*

La transmisión de la información genética entre generaciones y la expresión en forma de proteínas de dicha información genética no son suficientes para permitir que el programa de desarrollo de un organismo se realice correctamente. Además, es necesario asegurar que los genes se expresen en los momentos adecuados (cronológicamente o espacialmente bien) del desarrollo o bien como respuesta a cambios en las condiciones ambientales en las que se encuentra la célula o el individuo. Esto hace que el control de la expresión génica sea central en el proceso de la biología molecular de los seres vivos. Algo importante que se debe señalar, es que aunque existe una gran cantidad de DNA en los genomas de los organismos multicelulares no se transcribe todo. Las razones para ello son: hay una gran cantidad del genoma que no codifica para proteínas, existen diversos tipos de RNA, algunos genes se expresan únicamente en algunos momentos del ciclo celular y algunos genes únicamente se expresan en algunos tipos celulares. Esto es a pesar de que todas las células de un organismo multicelular contienen por lo menos una copia de todo el genoma de la especie. Por tales motivos, el principal objetivo de la *Biología del desarrollo* es entender cómo a partir de una única célula indiferenciada se origina un organismo pluricelular adulto con un patrón organizado. Para estudiar este proceso, la Biología del desarrollo utiliza modelos animales. Se ha demostrado que el uso de organismos modelo como peces, moscas, ranas y gusanos genera conocimientos que pueden ser aplicados de manera análoga al desarrollo de los mamíferos.

Estos modelos presentan ventajas técnicas. Por ejemplo, en *Drosophila* se conoce el genoma completo (Adams et al., 2000), su propagación y mantenimiento es relativamente fácil, de bajo costo y técnicamente accesible para hacer genética y transgénicos, y además, se cuenta con una gran cantidad de mutantes (Török et al., 1993; Rφrth 1996; Deák et al., 1997). Otra gran ventaja de *Drosophila* es su corto ciclo de vida, de manera que, en unas semanas, se puede obtener una gran cantidad de individuos en sus diferentes etapas del desarrollo.

1.9. El ciclo vital de *Drosophila melanogaster.*

Con el conocimiento del ciclo de vida de la mosca es fácil comprender el uso de *Drosophila* como modelo experimental y sus ventajas técnicas. El ciclo de vida de *Drosophila* es muy corto comparado con los mamíferos y otras especies. *Drosophila melanogaster* es un insecto holometábolo, es decir, presenta etapas larvarias y una etapa adulta separadas por una etapa de pupa, durante la cual experimenta una metamorfosis completa. El ciclo de vida de *Drosophila* dura aproximadamente 10 días a 25°C. El huevo de *Drosophila* se forma durante un período de aproximadamente 3 días y medio. Una vez fecundado, la madre lo deposita al exterior e inmediatamente inicia la embriogénesis. En esta etapa se determina la polaridad antero-posterior y más tarde la dorso-ventral (Ingham, 1988; Johnston y Nüsslein-Volhard, 1992). Después de 24 horas, el embrión eclosiona dando lugar a una larva que pasará por 3 etapas larvarias. Durante este período las células larvarias básicamente no proliferan; sin embargo, crecen en volumen debido a la endoreduplicación de su material genético (Edgar y Orr-Weaver, 2001). A los 5 días, la larva entra a la etapa de pupa y se inicia la metamorfosis. Durante esta etapa, la mayoría de los tejidos larvarios son lisados. Las estructuras adultas se formarán a partir de la reorganización de los discos imaginales o imagales y los histoblastos formarán la epidermis abdominal del adulto. Los discos imagales darán lugar a las estructuras epidérmicas de la cabeza, tórax y genitales externos del adulto. Por último la mosca eclosiona y a las pocas horas de vida se encuentra lista para copular e iniciar el nuevo ciclo de vida (Figura 6).

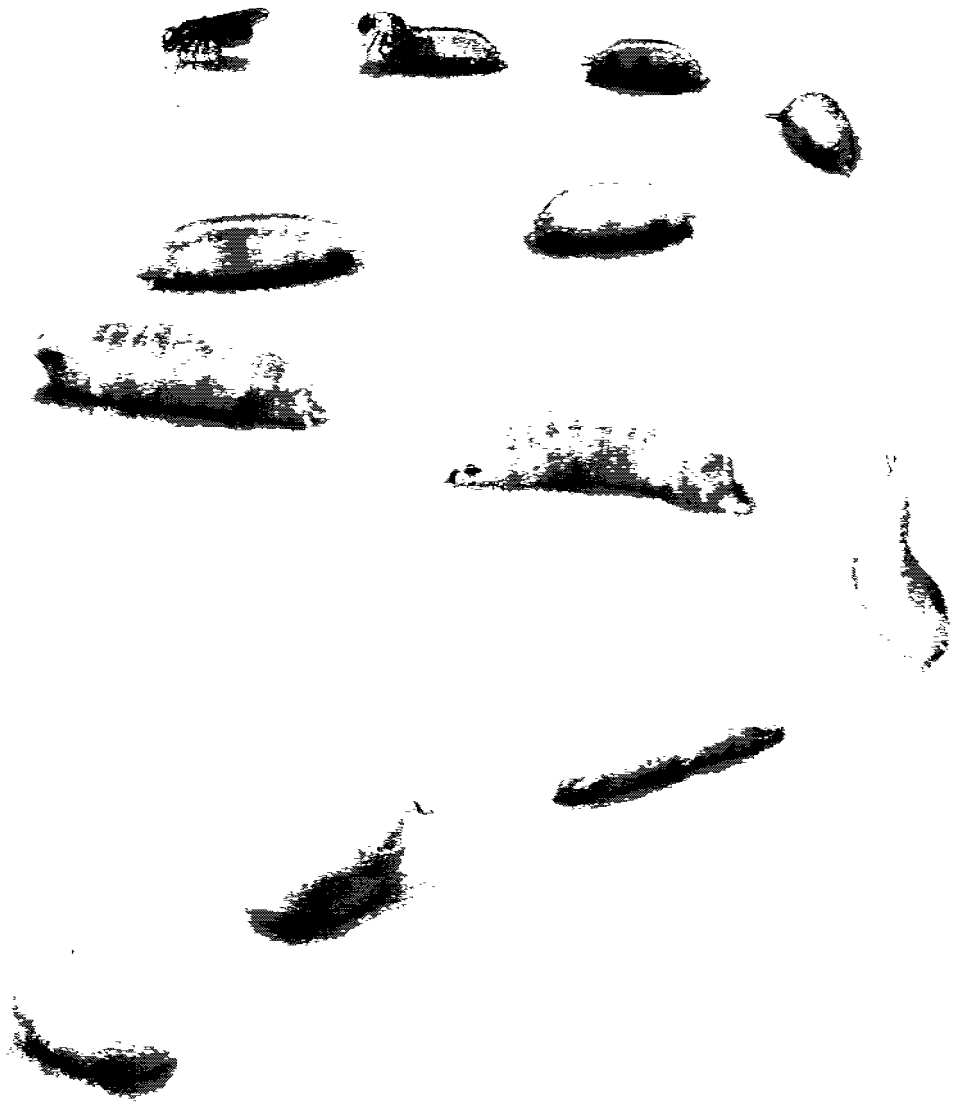

Figura 6. Ciclo de vida de *Drosophila melanogaster* (Tomado de Arbeitman et al., 2002).

1.10. Desarrollo embrionario de *Drosophila melanogaster.*

El desarrollo de un organismo comienza al darse la fertilización de un huevo por parte de un espermatozoide. En ese momento, cada célula dará origen a diferentes tejidos. También, el desarrollo embrionario está caracterizado por la formación de ejes. El embrión de *Drosophila* desarrolla un eje antero-posterior y un eje dorso-ventral. En la parte anterior del embrión se desarrolla la cabeza del adulto y en la parte posterior se forma el abdomen. Los ejes se establecen con los gradientes de mRNA y proteínas que se distribuyen

diferencialmente en el citoplasma. El mRNA y las proteínas son dotadas por la madre durante la ovogénesis. Esta dotación se conoce como herencia materna. Con la herencia materna el embrión no necesita sintetizar mRNA y proteínas para su sobrevivencia, al menos durante las primeras horas del desarrollo. El embrión en las primeras dos horas del desarrollo es un sincicio (blastodermo sincicial), los núcleos se dividen y migran en un citoplasma común. Los núcleos cigóticos se dividen rápidamente, en promedio cada 9 minutos, y sincrónicamente. Como resultado se tienen más de 8000 núcleos en la corteza del embrión. Al comienzo del ciclo catorce cesan las divisiones y la superficie de la membrana plasmática crece 30 veces y se invagina entre los núcleos, generando células independientes (Figura 7A). El evento de invaginación de la membrana celular para dar origen a las células del embrión es conocido como celularización (Figura 7B). La celularización de *Drosophila* equivale a la blástula media de *Xenopus* y del pez cebra (MBT). La MBT se caracteriza por la inducción de la expresión de genes cigóticos (Edgar y Schubiger, 1986). En la mosca, la transcripción cigótica inicia alrededor de 1.5-2h del desarrollo. Cuando el embrión está celularizado se considera que se encuentra en el estado de blastodermo celular (Foe et al., 1993). Posteriormente, la gastrulación da inicio. Esta etapa del desarrollo ocurre cuando un grupo de células ventrales de la blástula o blastodermo celular se invagina para crear un embrión de tres capas. Estos tres epitelios han sido llamados capas germinales: mesodermo, endodermo y ectodermo. El mesodermo dará lugar a los músculos, el endodermo formará el órgano digestivo y el ectodermo se diferenciará en epidermis, tejido nervioso central y periférico. En este momento, los genes del cigóto están siendo expresados, como los genes de segmentación, los cuáles controlan el número y la polaridad de los segmentos del embrión. También, da inicio la expresión de los genes homeóticos, los cuáles controlan la identidad de un segmento pero no afectan el número, la polaridad o el tamaño de los segmentos. Todos estos genes actúan para definir las partes de una mosca adulta (Browder et al., 1991).

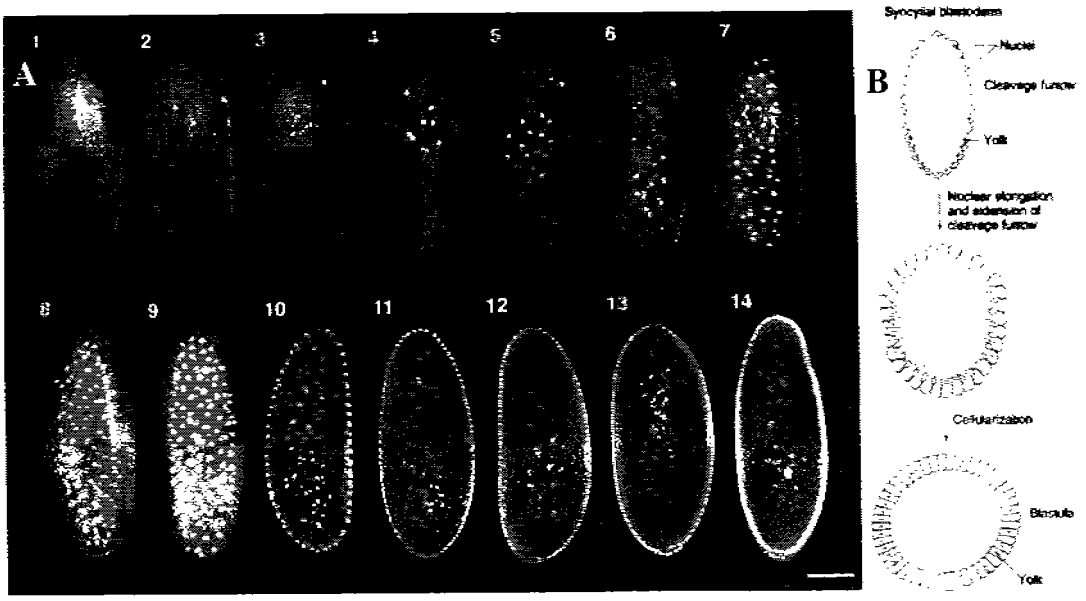

Figura 7. Desarrollo temprano del embrión de *Drosophila*. (A) Microscopía confocal de embriones silvestres de *Drosophila* teñidos con ioduro de propidio. La fotografía muestra las divisiones nucleares 1-13 del blastodermo sincicial y la interface de ciclo nuclear 14, celularización (*Drosophila* Protocols, 2000). (B) En el dibujo se observa el proceso de la celularización del embrión (1. Ver en las referencias de las páginas electrónicas).

1.11. El control genético de la segmentación en *Drosophila*.

Los insectos están compuestos por una serie de segmentos separados, los cuales están especializados en funciones específicas (identidad). Por ejemplo, el segmento torácico está especializado en la locomoción, y en el desarrollo de las alas y las patas. En *Drosophila,* la fertilización del huevo da lugar a una mosca adulta con segmentos que poseen identidad. El control genético de la segmentación en *Drosophila* implica una cascada de regulación de la expresión de los genes de la segmentación. La cascada da inicio con la difusión de factores maternos en el citoplasma, estos productos se conocen como genes maternos (por ejemplo: *bicoid*, *nanos*, *caudal*, etc). Estos genes se localizan a lo largo del eje anterior-posterior del embrión y controlan el patrón espacial de la transcripción de los genes "gap" (por ejemplo: *hunchback, Krüppel, knirps*, etc). Los genes "gap" son los primeros genes cigóticos que se expresan cuando se activa la transcripción del embrión. Estos genes codifican para factores de transcripción, y al igual que los genes maternos, los genes "gap" son expresados a lo largo del eje anterior-posterior del embrión de la mosca. Se expresan en un patrón no

periódico y en amplios dominios, cada dominio abarca varios segmentos continuos. Los genes "gap" regulan el siguiente grupo de genes en la jerarquía, los genes "pair-rule" (por ejemplo: *even-skipped*, *hairy*, *fushi tarazu*, etc). Los genes "pair-rule" son expresados en un patrón de 7 bandas repetidas. Estos genes codifican para factores transcripcionales que estabilizan la expresión de los genes de la polaridad del segmento (*wingless, hedgehog, engrailed,* etc). Estos últimos genes son expresados en un patrón de 14 bandas repetidas (Figura 8A). Los genes de la polaridad del segmento codifican para proteínas reguladoras como receptores, cinasas, etc. Estos genes median la interacción entre las células, determinan el número de segmentos y la parte anterior-posterior de cada uno de ellos. Al final de la cascada de regulación resulta una serie de parasegmentos y segmentos que tienen patrones repetidos idénticos de la expresión del gen de la polaridad del segmento. En seguida, los genes "pair-rule" y de la polaridad del segmento regulan a los genes homeóticos. Este último grupo de genes es requerido para asignar identidad a los diferentes segmentos del embrión, por ejemplo: cabeza, tórax, abdomen, etc (Figura 8B; Browder et al., 1991).

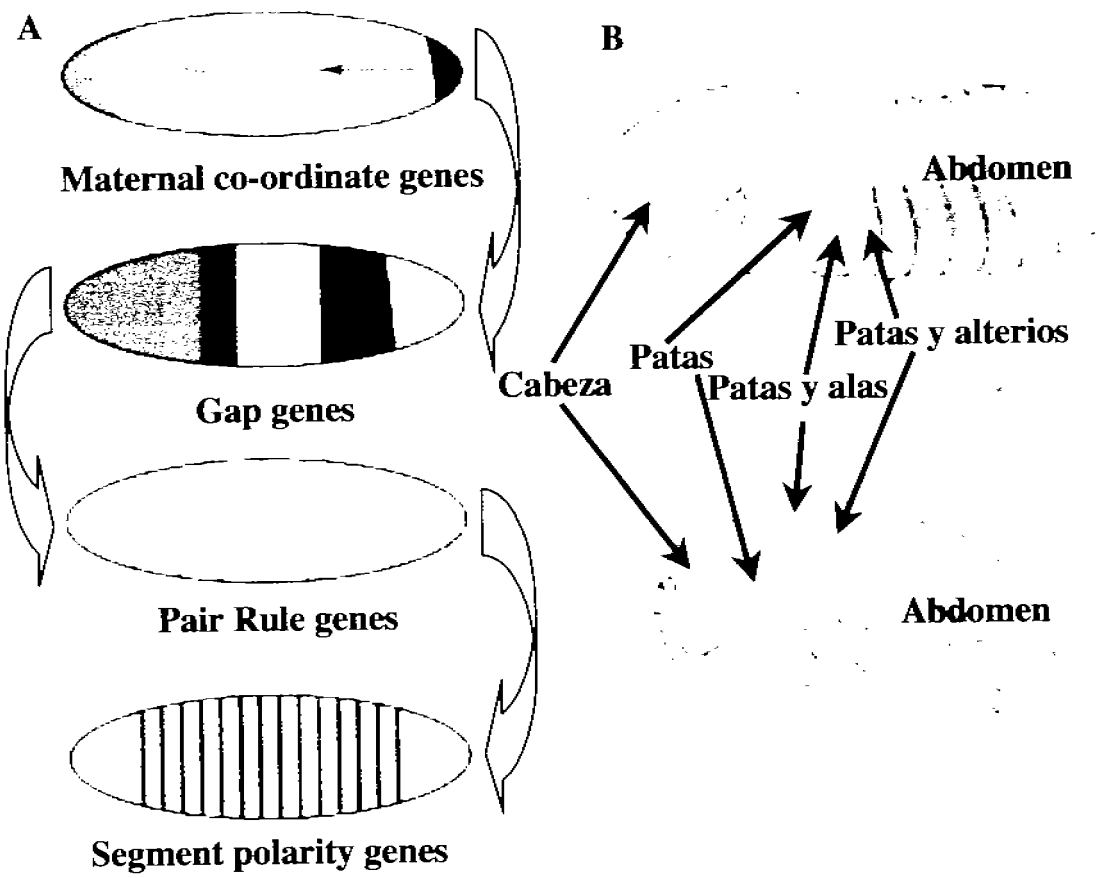

Figura 8. Control genético de la segmentación en *Drosophila.* (A) Cascada del control génetico de la segmentación de *Drosophila*. Los genes maternos se colocan en el sentido anterior-posterior del embrión. En seguida, los embriones se subdividen progresivamente en regiones por acción de cada grupo de genes de la segmentación (2. Ver en las referencias de las páginas electrónicas). (B) Una vez que los genes de segmentación dividieron al cuerpo en unidades repetidas, los genes homeóticos tienen la responsabilidad de formar estructuras específicas para cada unidad, dando así especialización o identidad a cada segmento (3. Ver en las referencias de las páginas electrónicas).

1.12. Hipótesis sobre la activación de la transcripción del genoma del embrión animal.

En muchos animales después de la fertilización, el genoma cigótico es transcripcionalmente inactivo y no requiere sintetizar mRNA (Yasuda y Schubiger, 1992). La transcripción se activa después de un número de ciclos celulares, este número es característico de cada especie (Thompson et al., 1998). Este mecanismo conservado parece tener un control de la activación transcripcional cigótica (ATC).

En el pez cebra, *Xenopus* y *Drosophila*, la ATC ocurre durante la transición de la blástula media (Zamir et al., 1997). Esta transición se caracteriza por el incremento del tiempo en el cual se lleva acabo el ciclo celular (Browder et al., 1991; Sibon et al., 1997). En *Drosophila*, esta transición toma lugar durante el establecimiento del blastodermo celular, 10-13ra división nuclear. Hasta la fecha no existe un principio que proponga cómo se da la activación de la transcripción del embrión. Pero, se ha sugerido que la duración del ciclo celular puede ser un punto de regulación. Ya que se piensa que la regulación negativa de la activación de la transcripción del genoma del embrión es debida a los períodos cortos de tiempo en los cuales se llevan acabo los ciclos celulares en el blastodermo sincicial. Con lo anterior, se propone que la transcripción es solamente posible cuando los ciclo celulares se retrasan. Lo anterior es apoyado con observaciones realizadas en ratón. En este último modelo animal, los ciclos celulares son muy largos, por lo que se argumenta que este período de tiempo da cabida a la activación de la transcripción. También, se propone que los factores maternos citoplásmicos podrían regular negativamente a la activación de la transcripción del embrión. En este último caso, la transcripción se dará solamente cuando el número de núcleos es correcto en un citoplasma común. Esta relación núcleo/citoplasma diluye y depleta a los factores maternos citoplásmicos negativos que reprimen la activación de la transcripción del embrión (Browder et al., 1991). Recientemente, se ha propuesto que la ATC responde a una regulación sobre la proteína BSF ("Bicoid Stability Factor") y el motivo CAGGTAG. Este heptámero de nucleótidos se encuentra localizado cuesta arriba y muy cercano al sitio de inicio de la transcripción de los genes activados durante el inicio de la transcripción del genoma cigótico de *Drosophila*. Un gran porcentaje de los genes que contienen este heptámero no tiene intrones, esto sugiere una ventaja para asegurar la producción funcional de transcritos en rondas de ciclos nucleares que ocurren muy rápido. Este heptámero funciona como un inductor de la transcripción ("enhancer"), ya que fusionado a un gen reportero (GFP) incrementó la cantidad del transcrito. Además, el heptámero CAGGTAG está ubicado entre secuencias moduladoras de la transcripción, las cuales son reconocidas por factores transcripcionales. Con lo anterior, se propone que el heptámero debe actuar en combinación con otros reguladores transcripcionales que participan en la ventana espacio-tiempo en la cual ocurre la ATC (De Renzis et al., 2007). Aún se desconoce la función para BSF, pero se sugiere que el homólogo en humano tiene el

papel de regulador transcripcional (Mancebo et al., 2001). Todo lo anterior sugiere que el motivo CAGGTAG y la proteína BSF participan en la activación de la transcripción cigótica.

Existen otras evidencias que sugieren el mecanismo que regula la activación de la transcripción del embrión. Como la existencia de un exceso de histonas maternas dentro del oocito de *Xenopus laevi*. Este exceso podría competir con el reclutamiento de la maquinaria basal de transcripción y controlar el inicio de la transcripción de los genes cigóticos (Prioleau et al., 1995). Por otro lado, se propone que en los primeros ciclos celulares del desarrollo embrionario del ratón existen rearreglos en la cromatina y cambios en la composición de proteínas nucleares, los cuales son probablemente los controladores de la ATC (Thompson et al., 1995). Un ejemplo de esto es la dinámica subcelular de la subunidad grande de la RNA polimerasa II (RPB 1) en el embrión. La cual es translocada gradualmente al núcleo durante el período de la ATC (Bensaude et al., 1983).

1.13. TFIIH en la embriogénesis temprana de *Drosophila*.

En el desarrollo temprano del embrión de *Drosophila*, la proteína DmXPD es depositada por herencia materna en el citoplasma, este producto también se expresa de manera ubicua durante el desarrollo de la mosca. Durante la embriogénesis temprana, DmXPD se localiza en el citoplasma del estado blastodermo sincicial y se moviliza a los núcleos del blastodermo celular. Esta dinámica subcelular coincide con un nivel bajo de la transcripción en el estado blastodermo sincicial y un nivel alto de la transcripción en el blastodermo celular, respectivamente. (Lamb y Laird, 1976; Foe et al., 1993; Reynaud et al., 1999). La transcripción de algunos genes específicos como histonas, "gap" y "pair rule", es detectada durante el ciclo mitótico diez del blastodermo sincicial (Edgar y Schubiger, 1986; Foe et al., 1993). Se sabe que en este momento la RNA polimerasa II está en el núcleo y todavía no es totalmente activa, ya que el dominio CTD no está fosforilado (Lecler et al., 2000). Es claro que la hiperfosforilación de la RNA pol II y los niveles altos de la transcripción de los genes cigóticos son observados al mismo tiempo que se forma el blastodermo celular, y es en esta ventana del desarrollo cuando DmXPD se encuentra totalmente en el núcleo (Reynaud et al., 1999). Estos datos sugieren una correlación entre la

localización nuclear de DmXPD y el inicio de la transcripción cigótica en el blastodermo celular de *Drosophila*.

2. JUSTIFICACIONES.

A.- Los componentes de TFIIH son proteínas esenciales para la célula y están altamente conservados en los eucariotes (Egly, 2001). Haywire de *Drosophila melanogaster* es la proteína homóloga a XPB de humano y DmXPD es la proteína homóloga a XPD. Estas proteínas son esenciales para la función de TFIIH en la REN y en la transcripción mediada por RNA pol II. Mutaciones en *haywire* provocan fenotipos en *Drosophila* parecidos a XP, SC y TTD (Merino et al., 2002). Todo esto sugiere que el desarrollo de la mosca es un buen modelo para el estudio de las funciones de TFIIH en la REN y en la transcripción. Así también, la generación de ratones transgénicos ha facilitado conocer las funciones de algunos genes de la maquinaria basal de transcripción y de reparación del DNA (De Boer y Hoeijmakers, 1999; van der Horst et al., 1997). En ratones nulos para los genes *xpd* y *xpb* se encontró que los embriones mueren antes de la etapa de dos células, en dicha etapa del desarrollo da inicio la transcripción cigótica (Thompson et al., 1995). Por esta evidencia, el ratón como modelo experimental ha proporcionado poca información sobre la participación de TFIIH en el inicio de transcripción cigótica. En este estudio proponemos al embrión de *Drosophila melanogaster* como un modelo experimental para entender la participación de TFIIH en procesos celulares y en particular su participación en la transcripción de los genes cigóticos.

B.- En muchos animales, el embrión en etapas tempranas es transcripcionalmente inactivo y no requiere de síntesis de mRNA para su desarrollo. Poco se sabe de cómo la maquinaria basal de transcripción es activada en el momento correcto del desarrollo temprano. Lo que se sabe es que los embriones de *Drosophila* tienen un nivel bajo de transcripción en el estadio de blastodermo sincicial y este nivel se incrementa después de la celularización (Lamb y Laird, 1976; Foe et al., 1993). También, se sabe que la transcripción de algunos genes específicos como el de las histonas, "gap" y "pair rule", es detectada durante el ciclo mitótico 10 del blastodermo sincicial (Edgar y Schubiger, 1986; Foe et al., 1993). En el ciclo mitótico 10, la RNA polimerasa II está en el núcleo y todavía no es totalmente activa, ya que el dominio CTD no está hiperfosforilado (Leclerc et al., 2000). Esto posiblemente se debe a dos razones: la primera, los niveles de fosforilación del CTD y de TFIIH son bajos y no pueden ser identificados con técnicas inmunológicas y segunda, que la RNA polimerasa

II no requiera del complejo TFIIH. Por estas razones en el presente trabajo pretendemos analizar la dinámica subcelular y la participación de un componente de la maquinaria basal de transcripción, TFIIH, en el inicio de la transcripción cigótica.

3. HIPÓTESIS.

Es un hecho que existe una correlación entre la localización nuclear de DmXPD y el inicio de la transcripción cigótica en el blastodermo celular de *Drosophila* (Reynaud et al., 1999). Por lo anterior, pensamos que los demás componentes de los subcomplejos "core" y CAK de TFIIH tienen una dinámica subcelular similar a DmXPD durante la activación de la transcripción de los genes cigóticos, dicha dinámica subcelular permite que se de una transcripción adecuada de los genes cigóticos durante la embriogenésis temprana de *Drosophila*.

4. OBJETIVOS.

4.1. Generales.

- Determinar la dinámica subcelular y del ensamblaje de los subcomplejos de TFIIH durante la activación de la transcripción de los genes cigóticos de *Drosophila melanogaster*.

4.2. Particulares.

- Determinar la dinámica subcelular de los subcomplejos "core" y CAK de TFIIH en el desarrollo temprano de la mosca.
- Analizar a los subcomplejos "core" y CAK de TFIIH sobre algunos promotores de genes que son transcritos durante la embriogénesis temprana de *Drosophila*.
- Determinar la dinámica del ensamblaje de los subcomplejos "core" y el CAK de TFIIH en el citoplasma y en los núcleos que inician la transcripción de los genes cigóticos.
- Determinar la función de las helicasas XPB y XPD en el "core" de TFIIH durante el inicio de la transcripción del embrión.

5. MATERIALES Y MÉTODOS.

5.1. Línea de mosca.

La línea w^{1118} (tipo silvestre) de *Drosophila melanogaster* fue usada para todos los experimentos del presente trabajo. Las moscas fueron mantenidas bajo condiciones estándares del laboratorio. La colecta de embriones fue realizada a 25°C en medios hechos con agar y jugo de manzana.

5.2. Inmuno-blot para detectar algunos componentes del "core" y CAK de TFIIH de *Drosophila melanogaster.*

En este ensayo se analizaron a las proteínas XPB y XPD, las cuales son componentes del "core" de TFIIH. Así también, se analizaron a las proteínas CDK7 y MAT1, las cuales son componentes del CAK de TFIIH. Los componentes del "core" y CAK fueron analizados en extractos de proteínas de embriones de la mosca. Para la detección de las proteínas se emplearon los anticuerpos específicos para cada una de ellas. Los extractos de proteínas de embriones de *Drosophila* fueron corridos en un gel SDS-PAGE. A continuación, las proteínas en el gel se transfirieron a una membrana de nitrocelulosa. La membrana con los extractos de proteínas se incubó en una solución bloqueadora (10% de leche descremada en PBS 1X (8 gramos (gr) de NaCl, 0.2 gr de KCl, 1.44 gr de Na_2HPO_4 y 0.24 gr de KH_2PO_4, pH 7.3) más 0.1% de Tween; PBST) durante una hora (hr) a temperatura ambiente (TA). En seguida, la membrana se lavó con PBST durante 5 minutos (min) y se incubó con los diferentes anticuerpos primarios durante una hr a TA. Los anticuerpos primarios fueron diluidos en una solución de PBST al 5% de leche descremada. Inmediatamente después, la membrana se lavó 3 veces con PBST, cada lavado duró 10 min a TA. Al termino de los lavados, la membrana se incubó con los anticuerpos secundarios acoplados a la enzima peróxidasa (HRP) durante una hr a TA. Los anticuerpos secundarios fueron diluidos en PBST al 5% de leche descremada. Transcurrido el tiempo, la membrana se lavó 3 veces con PBST, cada lavado duró 10 min. Al final, la membrana se lavó 2 veces con PBS, cada lavado duró 5 min. Por último, la presencia de las proteínas en la membrana fue revelada con diaminobenzidina (DAB) y peróxido de hidrógeno. También, la presencia de las proteínas fue detectada con el sistema de quimioluminiscencia (Lane, 1998).

5.3. Inmunotinción de TFIIH en embriones y glándulas salivales de *Drosophila*.

Los embriones de 0 a 4 hrs del desarrollo fueron colectados y decorionados con una solución de hipoclorito de sodio al 2.5%. En seguida, los embriones fueron fijados con una mezcla de formaldehído (FO) al 4% en PBS 1X-heptano (1:1), y desvitelinizados con metanol. Para iniciar el protocolo de inmunotinción, los embriones fueron hidratados con TBST (Tris-base 20mM, NaCl 150mM y Tween al 0.5%) y bloqueados con 10% de suero de cabra (Zymed) en TBST durante 9 hrs a temperatura ambiente. A continuación, los embriones se incubaron con los anticuerpos primarios a una dilución 1:1000 en TBST al 5% de suero de cabra toda la noche (O/N) a TA. El exceso de anticuerpo primario fue lavado con TBST. Los siguientes anticuerpos primarios fueron usados en experimentos de este tipo: haywire/XPB (Merino et al., 2002), DmXPD/XPD (Reynaud et al., 1999), hCDK7, hMAT1 y hTBP (Santa Cruz Biotechnology, Santa Cruz, CA). En seguida, se colocaron los anticuerpos secundarios a una dilución de 1:1500 en TBST al 5% de suero de cabra durante 2 hrs a TA. El exceso de anticuerpo fue lavado con TBST. Los anticuerpos secundarios empleados para este experimento fueron: anti-Fab-Cy2 y Cy3 para diferentes especies (Rockland). El DNA de los embriones fue teñidos con 15 nM de "Sytox Green" (Roche). Para teñir el DNA, los embriones fueron preincubados con RNAsa A (Roche) a una concentración de 10 μg/ml en TBST durante una hr a TA. Las inmunotinciones de las glándulas salivales de larvas del tercer estadio fueron realizadas de forma similar a las hechas en los embriones. Finalmente, las inmunotinciones de los embriones y de las glándulas salivales fueron montadas y visualizadas en un microscopio confocal.

5.4. Inmunotinciones de TFIIH en los cromosomas politénicos.

Los cromosomas politénicos fueron fijados y extendidos siguiendo el protocolo reportado por Engels (1986) y con modificaciones hechas por Reynaud et al (1999). En seguida, los cromosomas fueron hidratados con PBS 1X y bloqueados con 10% de leche en PBSI (PBS con 0.1% de IGEPAL, Sigma) durante 2 hrs a TA. Posteriormente, el anticuerpo primario fue añadido a una dilución 1:1000 en PBSI al 1% de leche e incubado O/N a 4°C. El exceso de anticuerpo fue lavado con PBSI. Después, los cromosomas fueron incubados con el anticuerpo secundario durante 2 hrs a TA. El anticuerpo secundario excedente fue lavado

con PBSI. Finalmente, las preparaciones fueron montadas y visualizadas en un microscopio confocal.

5.5. Inmunoprecipitación de cromatina (ChIP) y co-inmunoprecipitaciones (CoIP) de fracciones citoplásmicas y nucleares.

Embriones de 30 a 180 min del desarrollo fueron colectados y decorionados con 2.5% de hipoclorito de sodio durante 2-3 min a TA. En seguida, los embriones fueron fijados con 1.8% de FO en PBS 1X durante 15 min a TA. La fijación de los embriones fue detenida con lavados de PBST (PBS al 0.01% de Tween). Los embriones fijados fueron congelados en nitrógeno líquido y guardados a –70°C para su concervación (Orlando et al., 1997). Para la obtención de la cromatina, los embriones fueron descongelados y resuspendidos en la solución de lisis (1% SDS, 10mM EDTA, 50mM Tris, pH 8.0) con inhibidores de proteasas e incubados durante 10 min en hielo. Inmediatamente después, los embriones fueron sonicados hasta obtener fragmentos de cromatina entre 200 y 1000 pares de bases (pbs). Las muestras fueron centrifugadas a 14000*g* durante 10 min a 4°C. El sobrenadante extraído fue diluido 1:10 en la solución ChIP (0.01% SDS, 1.1% Tritón X-100, 1.2 mM EDTA, 167 mM NaCl, 0.1% de deoxicolato de sodio y 16.7 mM Tris-HCl, pH 8.0) con inhibidores de proteasas. El 5% del total de la cromatina sonicada (input) fue incubando a 65°C O/N. La cromatina total fue pre-limpiada con sefarosa-proteína G/A (Invitrogene). Para este experimento, la sefarosa-proteína G/A fue bloqueada previamente con 0.05% de BSA ("Bovine Serum Albumin"; Sigma) en solución ChIP. Por otra parte, el anticuerpo primario fue acoplado a la sefarosa-proteína G/A en la solución ChIP durante 3 hrs a 4°C. Los siguientes anticuerpos primarios fueron usados para este tipo de experimento: anti-DmCDK7 (Santa Cruz Biotechnology), anti-XPD, anti-DmXPB y el anti-CTD de la RNA pol II (Covance). Un anticuerpo primario irrelevante (anti-IgG o IgM) fue usado como control negativo. Después, la cromatina pre-limpiada y el anticuerpo primario acoplado a la sefarosa-proteína G/A fueron incubados durante 3 hrs a 4°C. Transcurrido el tiempo, el complejo sefarosa-anticuerpo-cromatina fue lavado 5 veces con solución ChIP, 2 veces con solución baja en sal (0.1% SDS, 1% Tritón X-100, 2mM EDTA, 150 mM NaCl y 20 mM Tris-HCl, pH 8.0), 2 veces con solución alta en sal (0.1% SDS, 1% Tritón X-100, 2mM EDTA, 500 mM NaCl y 20 mM Tris-HCl, pH 8.0), 2 veces con solución de LiCl (250 mM

LiCl, 1% IGEPAL, 1 % deoxicolato de sodio, 1 mM EDTA y 10 mM Tris-HCl, pH 8) y por último se lavó 2 veces con solución TE (10 mM Tris-HCl, pH 7.4 y 1 mM EDTA, pH 8). El complejo precipitado fue resuspendido en 100 µl de TE e incubado con RNAsa a una concentración de 50 µg/ml durante 30 min a 37°C. A continuación, la muestra fue ajustada a 0.5% de SDS y 0.5 mg/ml de proteinasa K (Roche) e incubada durante una hr a 45°C. Por último, el material precipitado fue incubado O/N a 65°C. Una vez hecho el tratamiento anterior al material precipitado, el DNA fue extraído con una solución de fenol/cloroformo (1:1) y precipitado O/N. El DNA extraído fue usado como templado para amplificar productos de 400 pbs por PCR. Los oligonucleótidos utilizados en este experimento se enuncian a continuación: promotor de *hunchback (hb)*; caagtgcgcataatttttg (oligo 5') y tgaaggcgatttgagtgatt (oligo 3'), exón de *hb*; acaccctgttacaactgcga (oligo 5') y cattcctggccatgtgaacg (oligo 3'), promotor de *Salivary gland secretion 5 (Sgs5)*; ggaacctgtgtgataaagct (oligo 5') y ctgaagagcccattggtagt (oligo 3'), exón de *alpha thalassemia/mental retardation syndrome X-linked (atrx)*; ccggggtaccgcatgcgtcgatctcgt (oligo 5') y ccgggtaccgcatgcatggccgagtac (oligo 3'), promotor de la histona H3 *(H3)*; ttggcgccacccttcccaa (oligo 5') y agccatctccgatttgggtt (oligo 3'). Los productos amplificados por PCR fueron analizados en geles de poliacrilamida y la señal se detectó en un film para radiografía.

Para los experimentos de co-inmunoprecipitación, las fracciones citoplásmicas y nucleares fueron preparadas de embriones colectados entre 1 y 2.5 hrs del desarrollo como describió Reynaud et al (1999). La fracción nuclear transcripcionalmente activa fue usada para este experimento (Kamakaka et al., 1991; Kamakaka y Kadonaga, 1994). Para descartar la contaminación de proteínas citoplásmicas en la fracción nuclear, realizamos ensayos de inmuno-blot con el objetivo de detectar a la enzima super-óxido-dismutasa 1 (SOD1), la cual es estrictamente citoplásmica. La CoIP fue hecha de acuerdo al protocolo descrito por Leclerc (1996). Los inmunoprecipitados nucleares y citoplásmicos obtenidos con los anticuerpos anti-DmXPB y anti-DmCDK7 fueron analizados en ensayos de inmuno-blot empleando los anticuerpos anti-XPB, anti-XPD, anti-DmCDK7 y anti-MAT1.

5.6. Neutralización de XPB y XPD por inyección de anticuerpos específicos e hibridación *in situ* para el gen *fushi tarazu (ftz).*

Se colectaron embriones en la etapa de blastodermo sincicial temprano, entre 0 y 30 min de ovoposición. Los embriones fueron decorionados y alineados sobre un portaobjetos, el cual tenía una cinta adhesiva doble cara. Esta preparación fue sumergida en aceite de halocarbono para prevenir la deshidración de los embriones. Luego, los embriones fueron microinyectados manualmente. Inyectamos cerca de 500 embriones por cada anticuerpo. Los anticuerpos inyectados en este experimento fueron los siguientes: anti-XPB, anti-XPD y anti-CDK7 (80 μg/ml para cada anticuerpo). Como control negativo inyectamos el anticuerpo contra la proteína verde fluorescente (anti-GFP). Los anticuerpos empleados en este experimento fueron purificados por afinidad a la proteína correspondiente. La preparación de los embriones y la microinyección fueron realizadas a 18°C. Después de la inyección, los embriones fueron incubados a 25°C durante 2.5 hrs. Una vez transcurrido el tiempo, el aceite de halocarbono fue lavado con heptano 100%. A continuación, los embriones fueron fijados con heptano 100%:37% formaldehído (1:1) durante 5 min. Por último, los embriones fueron procesados para realizar inmunotinciones o hibridaciones *in situ* para el gen *ftz*. Las imágenes de los experimentos de inmunotinción fueron colectadas con un sistema de microscopía confocal. Las hibridaciones *in situ* de los embriones inyectados fueron realizadas de acuerdo al protocolo estandar reportado por Mullen y DiNardo (1995).

6. RESULTADOS.

6.1. Dinámica subcelular del complejo TFIIH en el desarrollo temprano de la mosca.

Para determinar la dinámica subcelular de las proteínas del "core" y del CAK de TFIIH en la embriogénesis temprana de la mosca, lo primero que hicimos fue analizar la especificidad de los anticuerpos contra XPD/DmXPD, XPB/haywire, CDK7/DmCDK7 y MAT1 mediante ensayos de inmuno-blot. En este experimento observamos que los anticuerpos contra los componentes de TFIIH reconocieron una banda correspondiente a los pesos moleculares de XPD, XPB, CDK7 y MAT1 (Figura 9). A continuación, realizamos inmunotinciones de los componentes del CAK y "core" en los embriones tempranos de *Drosophila*. Lo que observamos fue que tanto XPB como XPD son depositados por dotación materna en el citoplasma (Figuras 10a y 11a). A medida que se dividen los núcleos y migran hacia la corteza del embrión, la señal de XPB y XPD se acumula en la periferia del embrión (Figuras 10b y 11b). En la división nuclear diez, XPB y XPD empiezan a localizarse dentro de los núcleos que se encuentran en la periferia, esto coincide con el inicio de la transcripción de algunos genes cigóticos (Figuras 10d, 10g, 11b y 11e). En la celularización, la cual ocurre entre la división nuclear trece y catorce, XPB y XPD se localizan preferencialmente dentro de los núcleos, en esta etapa el CTD de la RNA polimerasa II se encuentra fosforilado (Figuras 10e, 10h, 11c y 11f; Leclerc et al., 2000). Durante la gastrulación, ambas proteínas están localizadas en los núcleos que se encuentran en interfase, mientras que en células mitóticas, la señal de ambas proteínas se observa homogénea (Figuras 10f, 10i, 11d y 11g). Estos datos nos indican que XPB y XPD poseen una dinámica subcelular similar. Lo anterior fue corroborado al inmunoteñir a XPB y XPD en un mismo embrión (11h, 11i y 11j).

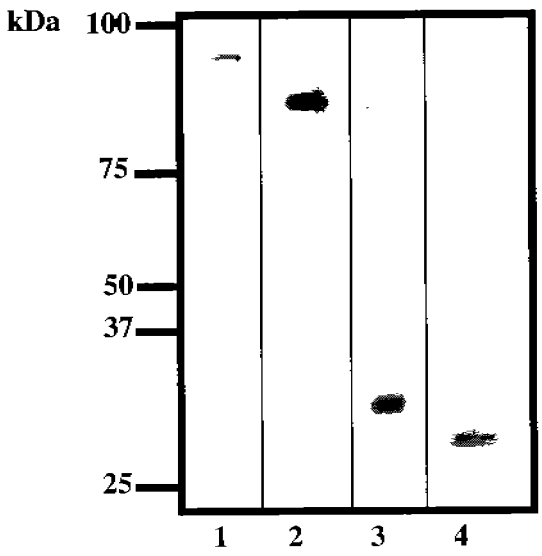

Figura 9. Detección de algunos componentes del "core" y del CAK de TFIIH en extractos totales de embriones de 0-20 hrs del desarrollo embrionario. Los extractos fueron analizados en geles SDS-PAGE y transferidos a membranas de nitrocelulosa. Las membranas fueron incubadas con los anticuerpos contra (1)XPB, (2)XPD, (3)CDK7 y (4)MAT1.

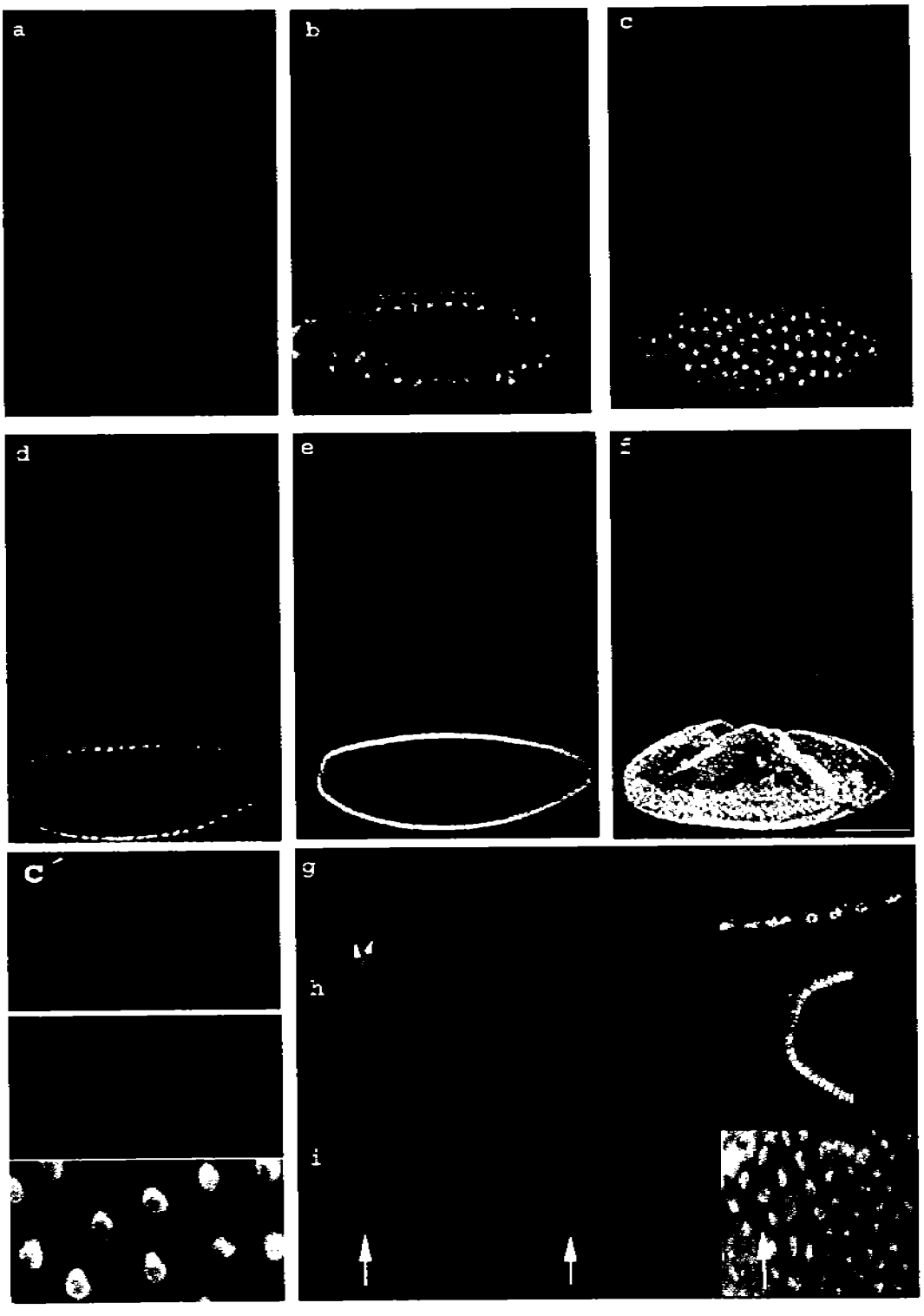
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Figura 10. Dinámica subcelular del "core" de TFIIH en el desarrollo temprano de la mosca. XPB en rojo y el DNA en verde. En cada figura se puede observar el empalme de la señal de XPB y el DNA. (a) Embrión en blastodermo sincicial, primera división nuclear. (b) Embrión en blastodermo sincicial, división nuclear 8. (c y c´) Superficie de un embrión en blastodermo sincicial, división nuclear 8. (d) Embrión en blastodermo sincicial, división nuclear 10. La señal de XPB se empieza a localizar en los núcleos que se encuentran en la periferia. (e) Embrión en blastodermo celular, la señal de XPB se localiza preferencialmente en el núcleo. (f) Embrión en la etapa de gastrulación. XPB está preferencialmente en los núcleos interfásicos y distribuido homogéneamente en las células mitóticas. (d´) Amplificación de la superficie de un embrión en la división nuclear 10. Las flechas indican la señal de XPB en el núcleo. (e´) Amplificación de un embrión en blastodermo celular. (f´) Amplificación de un embrión en gastrulación. Las flechas indican un grupo de cromosomas mitóticos.

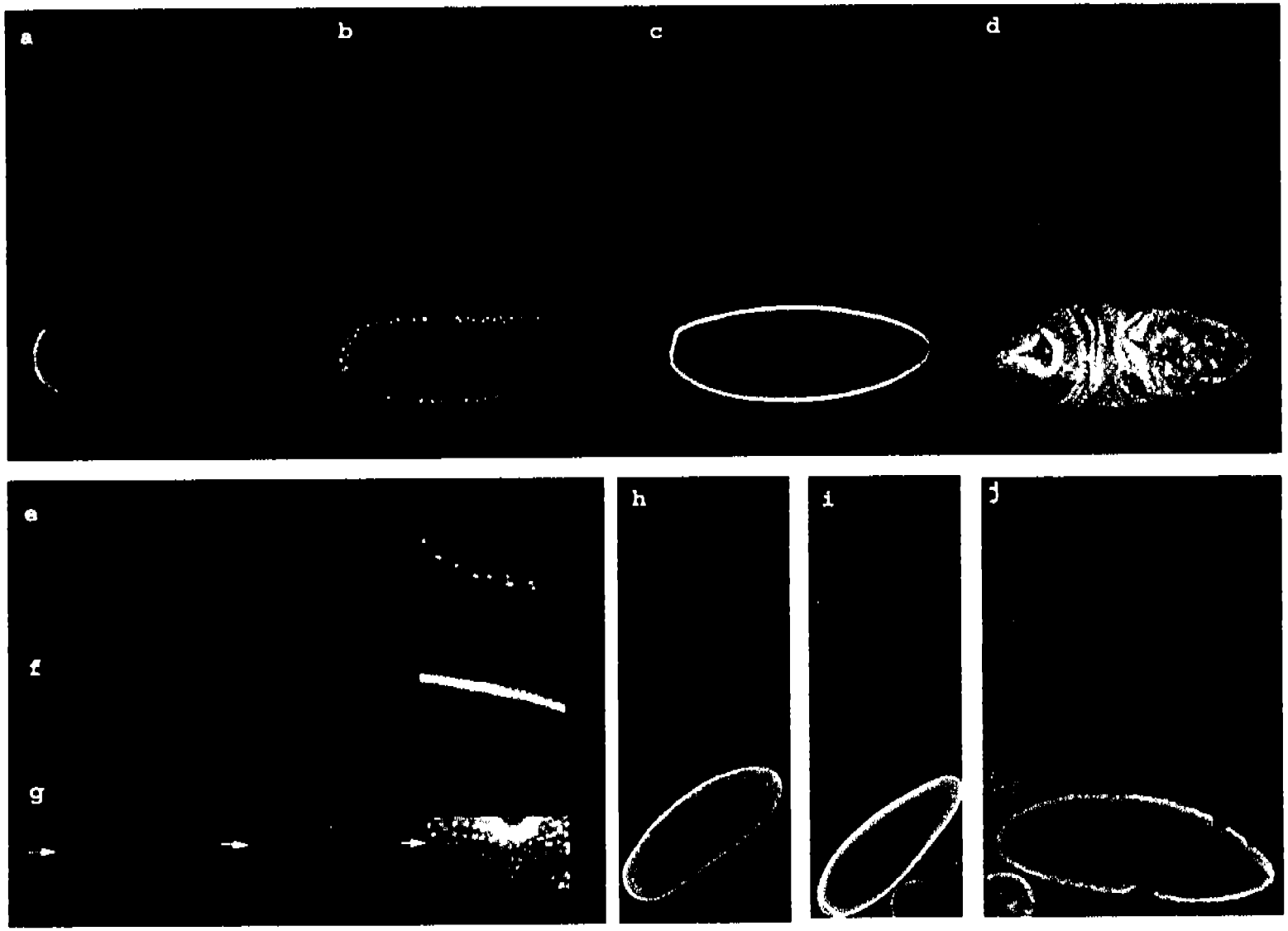
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Figura 11. Distribución de XPD y su co-localización con XPB en el embrión temprano de *Drosophila*. (a-g) XPD (rojo) y el DNA (verde) en el embrión de la mosca. En cada figura se puede observar el empalme de la señal de la proteína y el DNA. (a) Embrión en el ciclo mitótico 3 del blastodermo sincicial. XPD se observa en el citoplasma. (b) Embrión en el ciclo mitótico 10 del blastodermo sincicial. (c) Embrión celularizado. (d) Embrión en gastrulación. (e) Amplificación de una fotografía de un embrión en el ciclo mitótico 10. La señal de XPD se empieza a concentrar en el núcleo. (f) Amplificación de una fotografía de un embrión celularizado. XPD se observa preferencialmente en el núcleo. (g) Amplificación de una fotografía de un embrión en gastrulación. Las flechas indican la distribución homogénea y la señal reducida de XPD en un grupo de células en mitosis. (h-j) Co-localización de la señal de XPB (rojo) y XPD (verde) durante la embriogénesis.

En las inmunotinciones hechas para el CAK, encontramos que CDK7 y MAT1 tienen una distribución subcelular diferente a las proteínas del "core". Observamos que CDK7 y MAT1 son depositados por dotación materna en los embriones de la mosca (Figura 12a y 12e), al igual que las proteínas del "core". En la división nuclear diez, cuando los núcleos se encuentran en la periferia del embrión, la señal de CDK7 y MAT1 se detecta preferencialmente en el citoplasma (Figura 12b y 12f). Cabe señalar que en este momento del desarrollo las proteínas del "core" empiezan a acumularse en el núcleo. En la celularización y gastrulación, CDK7 y MAT1 se siguen localizando preferencialmente en el citoplasma (Figuras 12c, 12d, 12g y 12h). En resumen, las proteínas del CAK tienen una dinámica subcelular similar entre ellas, pero diferente a las proteínas del "core".

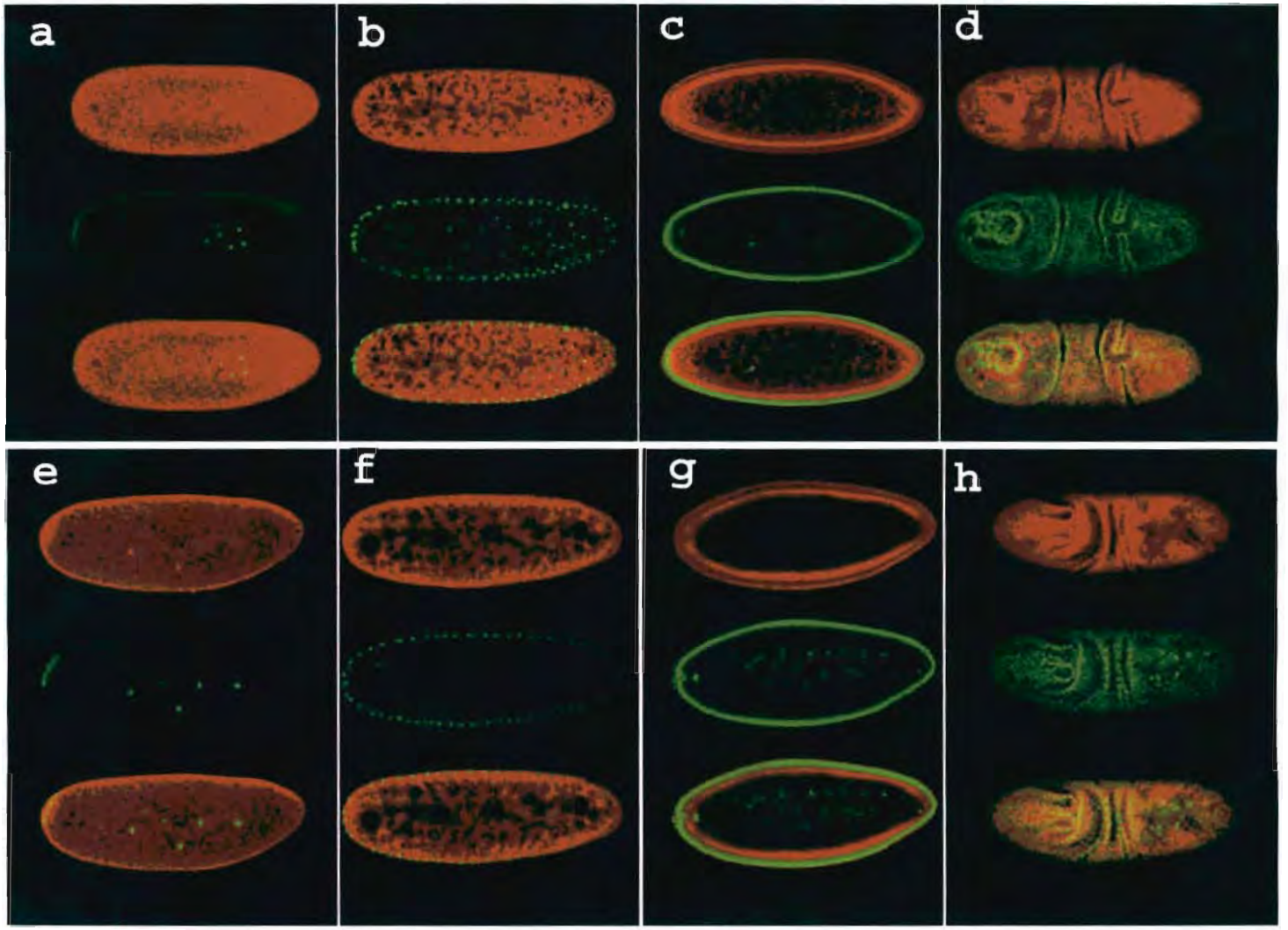
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Figura 12. Dinámica subcelular del CAK de TFIIH en el desarrollo temprano de *Drosophila.* CDK7 (a-d) y MAT1 (e-h) se muestran en rojo y el DNA en verde. En cada figura se puede observar el empalme de la señal verde y roja. (a y e) Embriones en blastodermo sincicial temprano. (b y f) Embriones en blastodermo sincicial, división nuclear 10. (c y g) Embriones en blastodermo celular. (d y h) Embriones durante la gastrulación. Las proteínas CDK7 y MAT1 están localizadas preferencialmente en el citoplasma durante todas las etapas del desarrollo. Ambas proteínas presentan una dinámica subcelular similar.

Con estos resultados nos quedó claro que el CAK y el "core" de TFIIH tienen dinámicas subcelulares diferentes durante la activación de la trancripción cigótica de *Drosophila.* Pero, aún así, corroboramos estos datos inmunotiñendo a XPD y CDK7 en un mismo embrión. Encontramos a XPD preferencialmente en el núcleo y CDK7 preferencialmente en el citoplasma durante la celularización (Figuras 13a y 13b) y gastrulación (Figuras 13a y 13c).

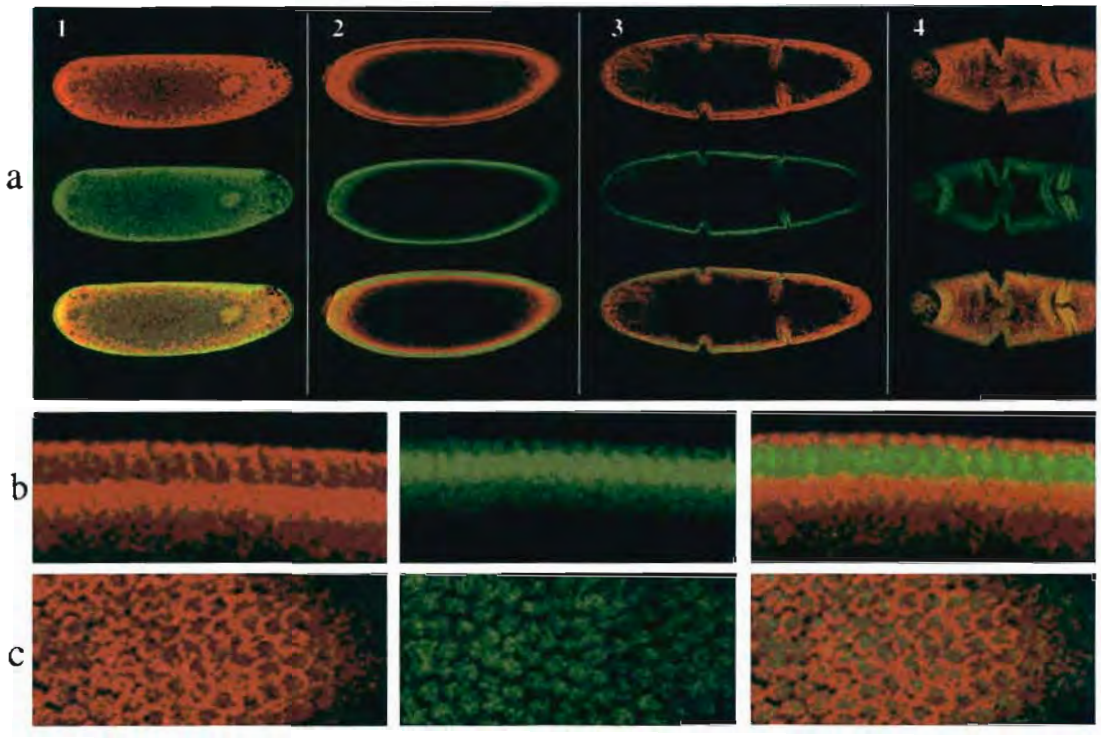


Figura 13. Co-tinción del "core" y del CAK de TFIIH en embriones tempranos de la mosca. (a) XPD (verde) está preferencialmente en el núcleo después de la división nuclear 13. CDK7 (rojo) está localizado preferencialmente en el citoplasma de embriones en blastodermo sincicial y celular. En cada figura se puede observar el empalme de la señal de XPD y CDK7. (a1) Embrión en

blastodermo sincicial temprano. (a2) Embrión en blastodermo sincicial, división nuclear 13. (a3 y a4) Embrión en gastrulación. (b) Amplificación de un blastodermo celular. (c) Amplificación de una gastrulación. La señal de XPD y CDK7 no co-localizan totalmente.

Sin embargo, los resultados de inmunotinción revelan que las proteínas del "core" también están en el citoplasma y que si hay una pequeña cantidad de las proteínas del CAK en el núcleo con esta técnica no es posible detectarla. Así que para saber con certeza la localización subcelular de ambos subcomplejos, realizamos ensayos de inmuno-blot para detectar a las proteínas XPB y CDK7 de fracciones citoplásmicas y nucleares. Estas fracciones fueron obtenidas homogenizando embriones en PBS 1X más inhibidores de proteasas, seguido de una centrifugación a 10000*g*. Encontramos que XPB está en la fracción nuclear y CDK7 en las fracciones citoplásmicas (Figura 14a). Así también, analizamos la presencia de estas proteínas en las fracciones citoplásmicas y nucleares obtenidas mediante el protocolo de extracción de fracción nuclear soluble (SNF; Kamakaka, 1991; Kamakaka y Kadonaga, 1994). La SNF contiene factores transcripcionales activos capaces de transcribir *in vitro*. En estos experimentos observamos que tanto XPB como CDK7 están en el núcleo y en el citoplasma de embriones celularizados (Figura 14b). Aunque estos resultados nos indican que el "core" es preferencialmente nuclear en este momento del desarrollo y el CAK es preferencialmente citoplásmico, sabemos que solamente la función del CAK en la transcripción puede ser observada cuando éste está unido al "core" en el núcleo. Por lo anterior decidimos analizar los subcomplejos CAK y "core" de TFIIH en un tejido diferenciado y transcripcionalmente activo, como son las glándulas salivales del tercer estadio larvario.

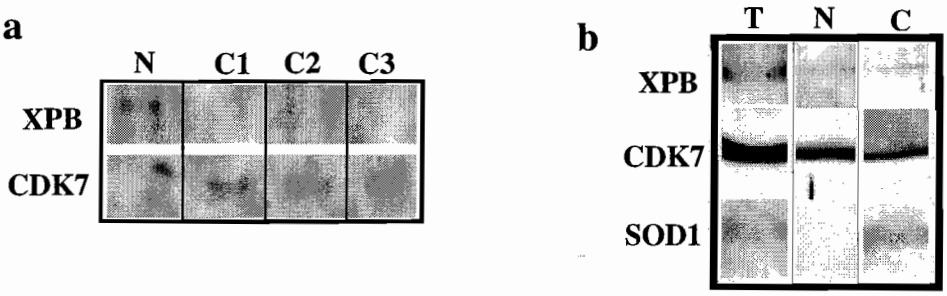


Figura 14. Identificación de las proteínas XPB y CDK7 en las fracciones nucleares y citoplásmicas de embriones en blastodermo celular, 2.5 hrs del desarrollo embrionario. (a) N representa la fracción nuclear y C1-3 las fracciones citoplásmicas. Nota: CDK7 fue encontrada en la fracción citoplásmica y XPB en la fracción nuclear. (b) En esta figura T representa el extracto total

de proteínas de embriones en blastodermo celular. SOD1 es una proteína citoplásmica usada como control. Nota: con este protocolo de extracción CDK7 y XPB fueron encontradas en el núcleo y en el citoplasma.

En las inmunoticiones de glándulas salivales encontramos que tanto las proteínas del CAK como las del "core" son preferencialmente nucleares (Figuras 15a y 15b). Por lo tanto, la distribución subcelular del CAK es diferente en las glándulas salivales del tercer estadio con respecto a la embriogénesis temprana. Interesantemente en preparaciones de cromosomas politénicos, CDK7 y XPD co-localizan en muchos sitios cromosomales, pero existen algunas regiones donde únicamente CDK7 es detectado con mayor intensidad (Figura 15c). Datos similares fueron observados cuando se realizaron las inmunotinciones de MAT1 y XPD (Figura 15d).

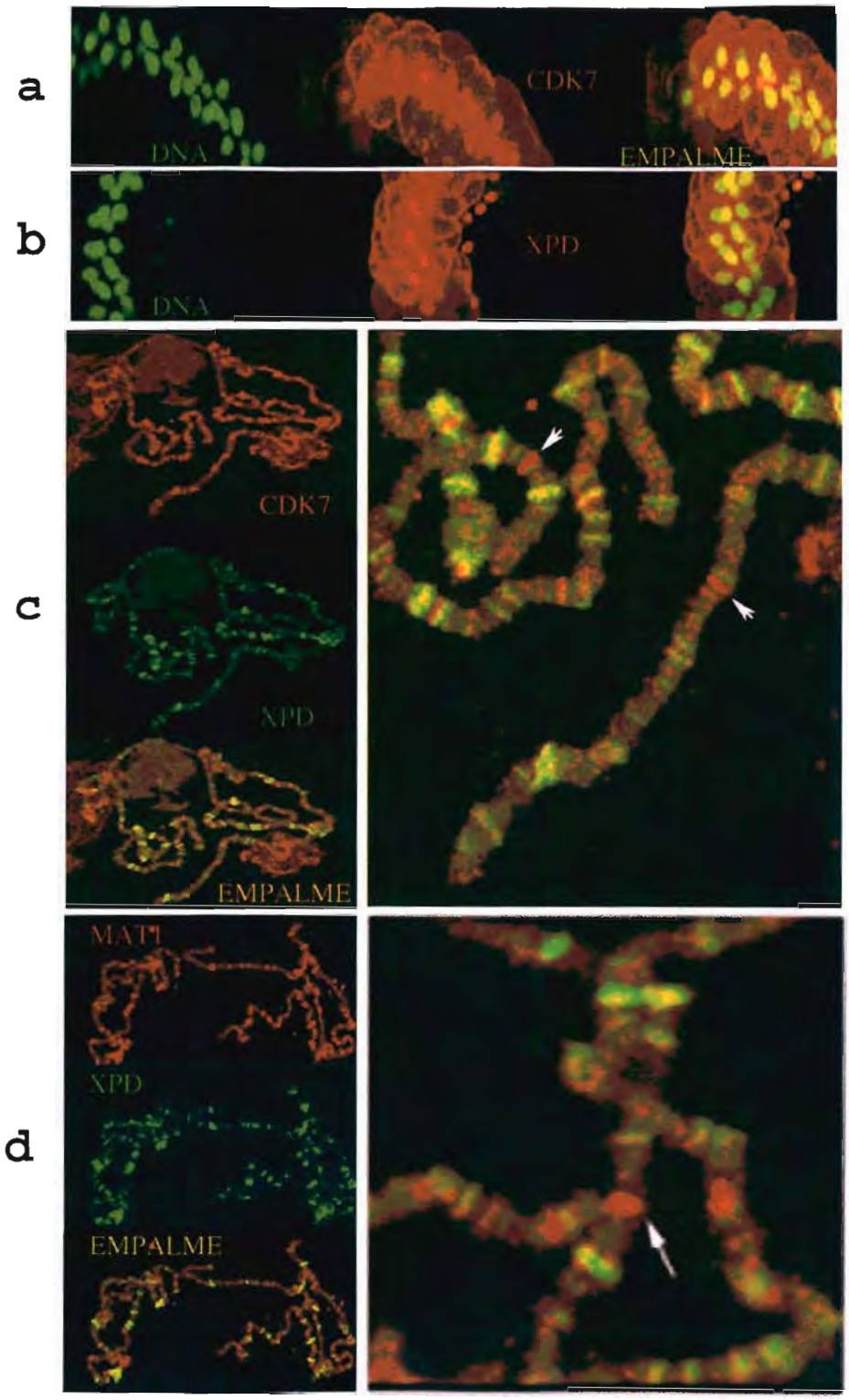

a
CDK7
DNA
EMPALME
b
XPD
DNA
c
CDK7
XPD
EMPALME
d
MAT1
XPD
EMPALME

Figura 15. Localización del "core" y del CAK de TFIIH en las células y en los cromosomas politénicos de las glándulas salivales de larvas del tercer estadio de *Drosophila*. (a) Inmunolocalización de CDK7 (rojo) y del DNA (verde). (b) Inmunolocalización de XPD (rojo) y del DNA (verde). (c) Co-tinción de CDK7 (rojo) y XPD (verde) en los cromosomas politénicos. (d). Distribución de MAT1 (rojo) y XPD (verde) en los cromosomas politénicos. En cada figura se puede observar el empalme de la señal verde y roja. El panel derecho de la figura c y d son amplificaciones de los cromosomas teñidos. Se observa que las proteínas del CAK y de XPD co-localizan en un patrón específico de eucromatina y la señal se enriquece en las regiones transcripcionalmente activas. Las flechas indican los sitios donde el CAK se encuentra posicionado sin XPD.

6.2. TFIIH se localiza en los promotores de genes que son transcritos durante la embriogénesis temprana de *Drosophila melanogaster.*

Los experimentos de inmunolocalización realizados en embriones tempranos de la mosca sugieren que el CAK de TFIIH, al localizarse preferencialmente en el citoplasma, no puede tener una función en la transcripción de los genes cigóticos. Pero, los experimentos de inmuno-blot sugieren que el CAK, el cual está en la SNF, si puede participar en el inicio de la transcripción. Los resultados de inmunolocalización del CAK contradicen una función muy importante para el TFIIH. Puesto que se sabe que TFIIH es indispensable para el inicio de la transcripción, tanto para formar el complejo abierto como para que la RNA pol II escape del promotor (Goodrich y Tjian, 1994). Para responder si CAK tiene un papel en la activación de los genes cigóticos, realizamos experimentos de inmunoprecipitación de la cromatina (ChIP) de algunos genes que se transcriben en las etapas de blastodermo sincicial tardio, durante y después del blastodermo celular. Para este ensayo empleamos anticuerpos contra DmCDK7, XPD, DmXPB y la RNA pol II. Seleccionamos los promotores de los genes *hunchback (hb)* e histona *H3* como genes que se transcriben durante el inicio de la transcripción del embrión. Como controles se analizaron: el promotor del gen *Sgs5*, el cual no se expresa en el embrión, un exón de *hb* y un exón del gen *atrx* de *Drosophila*, sobre los cuales ninguna de las proteínas de TFIIH debería estar (Figura 16a). Se sabe que *atrx* es un gen dotado por la madre como mRNA (Sun et al., 2006). En este experimento, encontramos a DmCDK7, XPD, DmXPB y la RNA pol II sobre los promotores de *hb* e histona *H3*. Únicamente la RNA pol II fue encontrada en el exón de *hb* y en el promotor de la histona

H3. Solamente la 'RNA pol II fue detectada sobre el promotor de *Sgs5*. Por último, ninguna de las proteínas analizadas fueron encontradas en el exón de *atrx* (Figura 16b). Con estos resultados podemos concluir que tanto el CAK y el "core" de TFIIH están sobre los promotores de los genes que se transcriben durante la embriogénesis temprana.

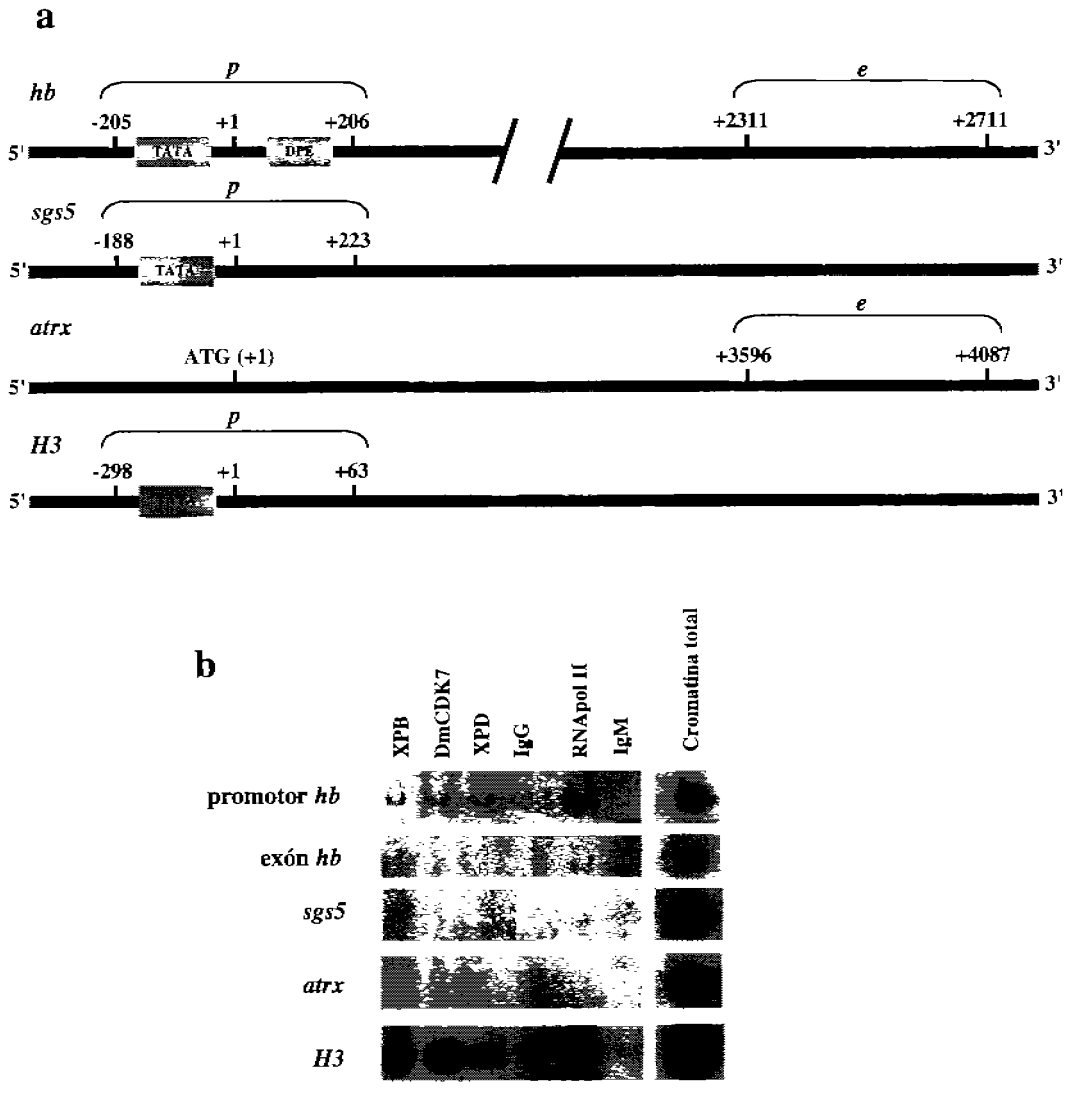


Figura 16. TFIIH se encuentra en los promotores de genes que son transcritos durante la embriogénesis temprana de *Drosophila melanogaster*. (a) Regiones amplificadas de los diferentes genes evaluados. El paréntesis abierto indica el producto generado por PCR, *p* = promotor, *e* = exón, TATA = caja TATA, DPE = elemento promotor cuesta arriba. (b) Fragmentos de cromatina de embriones de 30 a 180 minutos del desarrollo fueron inmunoprecipitados con los anticuerpos contra DmCDK7, XPD, XPB y RNA pol II. Dos anticuerpos controles negativos fueron empleados:

IgG e IgM. Las regiones inmunoprecipitadas fueron amplificadas por PCR. Como un control positivo de PCR, se amplificaron todas las regiones promotoras y de exones de los genes a partir de cromatina total.

6.3. El "core" y el CAK de TFIIH se ensamblan en los núcleos que inician la transcripción de los genes cigóticos.

Los experimentos de ChIP indican que el "core" y el CAK de TFIIH se encuentran sobre los promotores de los genes que se activan en el inicio de la transcripción cigótica, pero estos resultados no indican si los subcomplejos interactúan físicamente. Para determinar, cómo es la interacción entre CAK y el "core" durante la activación de los genes cigóticos, realizamos ensayos de co-inmunoprecipitación (CoIP). Los anticuerpos utilizados para este ensayo fueron el anti-CDK7 y anti-XPB. Estos anticuerpos fueron incubados con las fracciones citoplásmicas o nucleares (SNF) obtenidas a partir de embriones en la etapa de celularización. Con las co-inmunoprecipitaciones realizamos ensayos de inmuno-blot empleando los anticuerpos contra XPB, XPD, CDK7 y MAT1. Observamos que en extractos citoplásmicos, CDK7 no co-inmunoprecipita con XPB, pero sí con MAT1 y XPD (Figura 17, Panel Fracción Citoplásmica-CoIP CDK7). Este dato sugiere la existencia del complejo transitorio CAK-XPD en el citoplasma. Por lo contrario, XPB co-inmunoprecipita con XPD, pero no con CDK7 ni MAT1 (Figura 17, Panel Fracción Citoplásmica-CoIP XPB). Esto indica la presencia del "core" de TFIIH en esta fracción citoplásmica. Estos datos sugieren que TFIIH no está ensamblado en el citoplasma. En las fracciones nucleares: XPB, XPD y MAT1 sí co-inmunoprecipitan con CDK7 (Figura 17, Panel Fracción Nuclear-CoIP CDK7). Este dato demuestra que el CAK y el "core" están ensamblados formando a TFIIH. En resumen, parece ser que el TFIIH como complejo se encuentra en la regiones promotoras de los genes cigóticos analizados en los ensayos de ChIP. Estos datos están de acuerdo con el hecho de que TFIIH se necesita para la formación de complejo abierto y para el escape del promotor en la transcripción realizada por la RNA pol II (Dahmus, 1995; Bradsher et al., 2000).

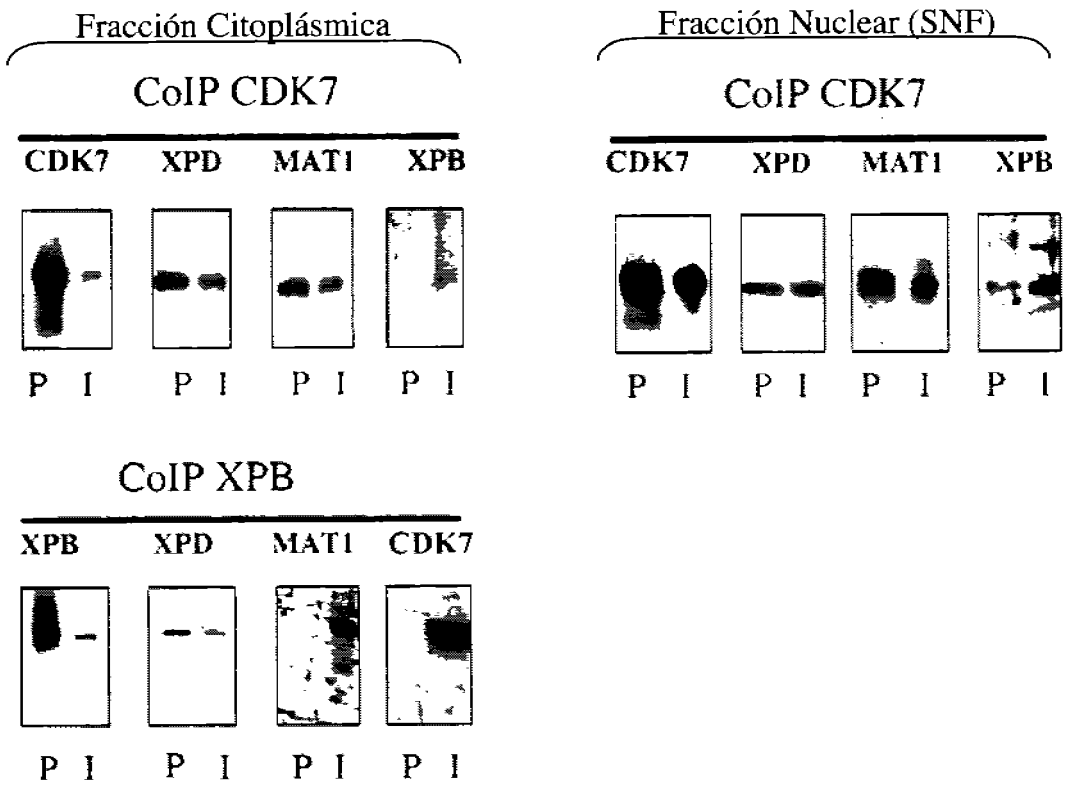


Figura 17. El "core" y el CAK de TFIIH se ensamblan en los núcleos que inician la transcripción de los genes cigóticos. Se colectaron fracciones citoplásmicas y nucleares (SNF) de embriones con un tiempo del desarrollo de 1 a 2.5 hrs. Los anticuerpos anti-CDK7 y anti-XPB fueron incubados con las fracciones citoplásmicas o nucleares (SNF). El material co-inmunoprecipitado y las proteínas totales de las fracciones celulares fueron analizadas en geles SDS-PAGE. Las proteínas se transfirieron a una membrana de nitrocelulosa y se analizaron en experimentos de inmuno-blot usando los siguientes anticuerpos: DmCDK7, MAT1, XPB y XPD.
P = CoIP e I = proteínas totales de la fracción citoplásmica o nuclear.

6.4. El ensamblaje de XPB y XPD en el "core" de TFIIH se requiere para que el "core" entre al núcleo durante el inicio de la transcripción del embrión.

Los experimentos de CoIP de las fracciones nucleares y citoplásmicas de embriones tempranos indican que el complejo CAK interacciona con XPD en el citoplasma formando el complejo transitorio CAK-XPD y que el CAK está unido al "core" formando a TFIIH en el núcleo. Así también, los datos de CoIP sugieren que el "core" está ensamblado en el citoplasma y posiblemente es transportado al núcleo durante el inicio de la transcripción

cigótica. Para determinar la importancia que tienen XPB y XPD en el transporte nuclear del "core", realizamos experimentos de neutralización de las proteínas del "core". Para esto se colectaron embriones de 0 a 30 minutos del desarrollo, los cuales fueron inyectados con los anticuerpos contra XPB, XPD, CDK7 y GFP (este último como control). Después de la inyección, los embriones se dejaron desarrollar entre las etapas de celularización y gastrulación, y posteriormente se inmunotiño el DNA, XPB, XPD y TBP ("TATA Binding Protein" como control). En embriones inyectados con el anticuerpo anti-XPD, la señal para XPB se observó en el citoplasma durante la etapa de blastodermo celular. Lo anterior fue observado únicamente en la mitad del embrión, mientras que en la otra mitad, la señal para XPB fue detectada tanto en el núcleo como en citoplasma de cada célula (Figura 18, panel ANTI-XPD). Algo similar fue observado cuando se analizaron los embriones inyectados con el anticuerpo anti-XPB. En estos embriones, la señal para XPB únicamente fue nuclear en la mitad del embrión en la etapa de blastodermo celular, mientras que en la otra mitad del embrión la señal para XPB no fue detectada (Figura 18, panel ANTI-XPB^3). En cambio, en embriones inyectados con el anticuerpo anti-XPB, la señal para XPD se observó en el citoplasma de cada célula de la etapa de gastrulación (Figura 18, panel ANTI-XPB^1). Este fenómeno de neutralización de la proteína XPB (paneles anti-XPD y anti-XPB^3) con respecto a la proteína XPD (panel anti-XPB^1), puede ser debido al establecimiento de un gradiente de neutralización de la proteína XPB con respecto a la etapa desarrollo embrionario. Ya que se ha demostrado que el material microinyectado se difunde a partir del sitio de microinyección a medida que transcurre el desarrollo del embrión de *Drosophila* (Kumar et al., 2001). En nuestro caso, la densidad de los núcleos observados en los embriones de los paneles anti-XPD y anti-XPB^3 nos indica que estos embriones fueron fijados en etapas del desarrollo más tempranas que el embrión del panel anti-XPB^1, donde se observa que la proteína XPD está preferencialmente en el citoplasma. Por otra parte, la inyección del anticuerpo anti-GFP no modificó la localización nuclear de XPB y XPD (Figura 18, panel ANTI-GFP). Mientras que en embriones inyectados con el anticuerpo anti-CKD7, se observaron núcleos únicamente en mitosis, además, los cromosomas mitóticos presentaron aberraciones (Figura 18, panel ANTI-CDK7). Como control podemos observar que la inyección de los anticuerpos anti-XPD (dato no mostrado) y anti-XPB no afectó la localización nuclear de TBP (Figura 18, panel ANTI-XPB^2). Este

experimento nos permitió comprobar que los datos observados con anterioridad fueron específicos para las subunidades de TFIIH. También, que XPB y XPD están ensamblados en el citoplasma y que probablemente ambas proteínas migran juntas al núcleo. Por lo tanto, a partir de estas evidencias, los datos de CoIP y las imágenes de microscopía confocal podemos sugerir un modelo de la dinámica subcelular y del ensamblaje de los subcomplejos de TFIIH durante el inicio de la transcripción cigótica. En este modelo se puede describir que el ensamblaje del "core" ocurre en el citoplasma de los embriones tempranos de *Drosophila*. Después, el "core" migra al núcleo durante el inicio de la transcripción cigótica. En el núcleo, el "core" se une al complejo transitorio CAK-XPD para formar a TFIIH (Figura 20).

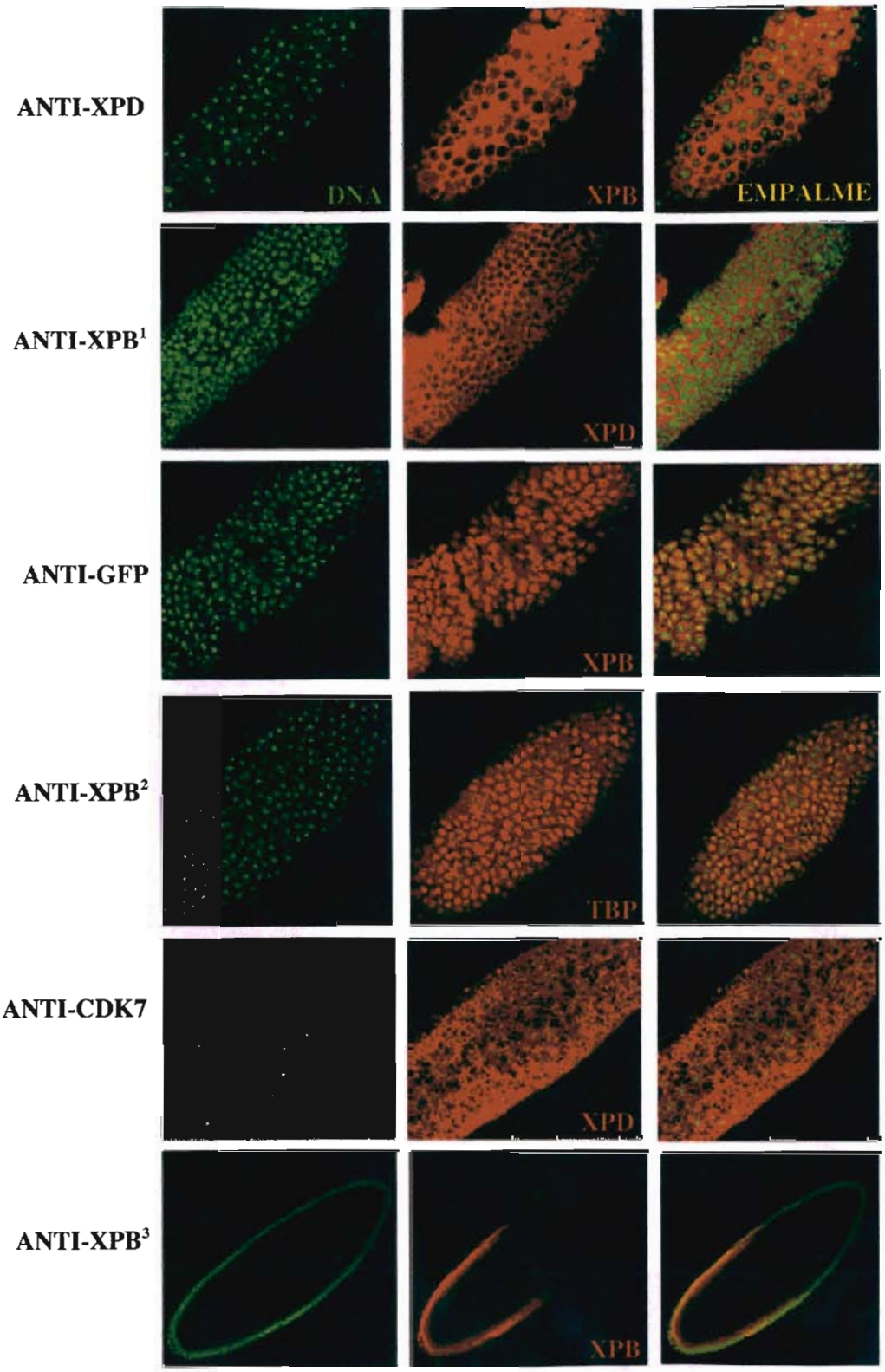

ANTI-XPD
DNA
XPB
EMPALME
ANTI-XPB1
XPD
ANTI-GFP
XPB
ANTI-XPB2
TBP
ANTI-CDK7
XPD
ANTI-XPB3
XPB

Figura 18. El ensamblaje de XPB y XPD en el "core" de TFIIH se requiere para que el "core" entre al núcleo durante el inicio de la transcripción del embrión. Los nombres a la izquierda de cada composición indican el anticuerpo inyectado. El DNA se muestra en color verde. La inmunotinción de las proteínas XPB, XPD, y TBP se observan en rojo. A la derecha de cada panel se puede observar el empalme de la señal verde y roja.

6.5. La inactivación de XPB o XPD afecta la expresión del gen *fushi tarazu (ftz)* en el inicio de la transcripción cigótica.

El experimento de neutralización tanto para XPB como para XPD nos indica que el ensamblaje de XPB y XPD en el "core" de TFIIH se requiere para que el "core" entre al núcleo durante el inicio de la transcripción del embrión. Es muy probable que la retención del "core" en el citoplasma afecte otras funciones importantes de TFIIH. Una de las funciones de vital importancia para el embrión que pudiera ser afectada es la transcripción de genes que se emplean para el desarrollo. (Leclerc, 2000). Por tal motivo, se evaluó la expresión del gen *ftz* durante el inicio de la transcripción cigótica mediante ensayos de hibridación *in situ* en embriones silvestres microinyectados y no microinyectados con los anticuerpos contra XPB y XPD. Elegimos evaluar la expresión de *ftz* porque es un gen que se expresa con un patrón canónico durante el inicio de la transcripción del embrión de la mosca (Figura 19a). En este experimento se observó que el patrón canónico del gen *ftz* fue afectado en los embriones microinyectados con los anticuerpos contra XPB o XPD (Figuras 19b y 19c, respectivamente). Por lo tanto, estos datos denotan que la neutralización de XPB o XPD afecta el inicio de la transcripción cigótica en el embrión de *Drosophila*.

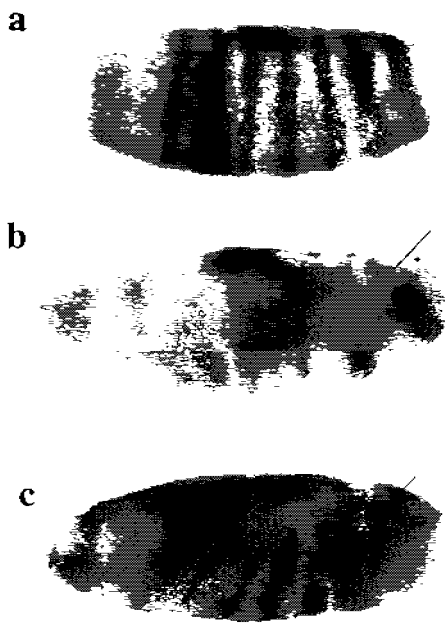


Figura 19. La neutralización de XPB o XPD afecta la expresión del gen *fushi tarazu* (*ftz*) en el inicio de la transcripción cigótica. Embriones de *Drosophila* de 0 a 30 min del desarrollo fueron inyectados con los anticuerpos específicos para las proteínas XPB o XPD. Después de 2.5 hrs del desarrollo, los embriones fueron fijados y sometidos al protocolo de hibridación *in situ* para identificar la expresión del mRNA de *ftz*. (a) Expresión típica del mRNA de *ftz* en un embrión silvestre. (b) Expresión del mRNA de *ftz* en un embrión microinyectado con el anticuerpo anti-XPB. (c) Expresión del mRNA de *ftz* en un embrión microinyectado con el anticuerpo anti-XPD. Las flechas indican la pérdida de expresión del mRNA de *ftz* en algunas células del embrión.

7. DISCUSIÓN.

7.1. El CAK y el "core" tienen distribuciones diferentes a nivel celular durante el desarrollo de la *Drosophila.*

En el presente trabajo encontramos que el CAK se encuentra preferencialmente en el citoplasma durante el desarrollo temprano del embrión de *Drosophila* y que es preferencialmente nuclear en células diferenciadas, como son las células de las glándulas salivales. Sin embargo, una fracción del CAK está unida al "core" sobre los promotores de genes que se transcriben durante la activación de la transcripción cigótica. Lo anterior no es extraño, ya que se sabe que TFIIH tiene tres funciones fundamentales en la célula: reparación del DNA, transcripción y control del ciclo celular. Con lo anterior, sugerimos que nuestros resultados muestran la participación de TFIIH en el inicio de la transcripción cigótica de *Drosophila.* Además, proponemos que el CAK participa probablemente en el control del ciclo celular en el citoplasma de los embriones, debido a que los complejos CAKs fosforilan a las Cdks, las cuales regulan la progresión del ciclo celular (Morgan, 1995). En el caso particular de CDK7, se sabe que se requiere para la activación de cdc2/Ciclina A y cdc2/ciclina B en *Drosophila* (Larochelle et al., 1998; Larochelle et al., 2001). Por lo que es probable que el CAK de TFIIH regule la actividad de cdc2 vía fosforilación en el citoplasma del embrión.

La presencia del CAK-XPD en el citoplasma sugiere un papel de CDK7 en la regulación del ciclo celular. La observación de anomalías en el ciclo celular al neutralizar con anticuerpos a CDK7 en el citoplasma de los embriones en blastodermo sincicial sugiere la función de CDK7 en el control del ciclo celular. Lo anterior es apoyado con la observación de la actividad de cinasa de CDK7 en la fase M del ciclo celular (Larochelle et al., 1998). Con lo anterior, sugerimos que la fracción del CAK que no interacciona con XPD posiblemente tiene funciones en el control del ciclo celular. Ya que al neutralizar a la proteína XPD o XPB no observamos anomalías en el ciclo celular del embrión. En otro laboratorio, se observó que la sobre-expresión de XPD regula la actividad de CDK7 en el control del ciclo celular del embrión de *Drosophila.* Estos datos hacen pensar que existen niveles diferentes de los componentes del "core" (Chen et al., 2003). Pero, en nuestro laboratorio, observamos que el patrón de expresión de XPB y XPD es muy similar y no varían los niveles de ambas señales a lo largo del desarrollo temprano del embrión de

Drosophila. Así también, observamos que el embrión no requiere de XPD y XPB para llegar al estado de gastrulación.

Por otra parte, pensamos que los niveles altos de transcripción que ocurren en las células de las glándulas salivales hacen que casi todo el CAK se modifique y se concentre en el núcleo. Sugerimos que las modificaciones postraduccionales en el dominio T-loop podrían estar regulando esta dinámica subcelular. El dominio T-loop modula la actividad de las Cdks (Jeffrey et al., 1995; Russo et al., 1996; Larochelle et al., 2001). Además, se ha reportado por otros grupos que tanto la forma fosforilada y no fosforilada de CDK7 están presentes durante el desarrollo embrionario de la mosca. Así también, se reportó que solamente la forma fosforilada de CDK7 está presente en las glándulas salivales de las larvas del tercer estadio (Larochelle et al., 2001). En nuestros experimentos con este mismo tejido, pero en preparaciones de cromosomas politénicos, el CAK y "core" co-localizan en muchos lugares de la cromatina y es más evidente en los lugares cromosomales activos en transcripción ("puffs"). Lo anterior no es extraño, ya que los dos subcomplejos se requieren para participar en la transcripción (Lu et al., 1992; Rossignol et al., 1997). Sin embargo, es sorprendente que en algunas regiones cromosomales únicamente está presente el CAK y no el "core". Este dato nos indica una función desconocida del CAK. Probablemente, el CAK sin el "core" regula la transcripción de algunos loci, ya que se sabe que CDK7 y la fosforilación de la serina 5 del CTD de la RNA pol II co-localizan a lo largo de los cromosomas politénicos de la mosca (Schwartz et al., 2003). Sin embargo, lo anterior no necesariamente indica una transcripción activa, puesto que la presencia de los factores generales de la transcripción sobre el promotor de un gen no refleja un estado activo de transcripción. Por ejemplo: la TBP, TAFs y TFIIB se encuentran sobre el promotor del gen *hsp70* de *Drosophila* antes de inducir su expresión con calor ("heat shock"), y después de exponer al "heat shock", la RNA pol II es reclutada al promotor *hsp70* (Lebedeva et al., 2005).

7.2. La interacción de XPB y XPD en el "core" de TFIIH se requiere para que el "core" se transporte del citoplasma al núcleo y para activar la transcripción de los genes cigóticos.

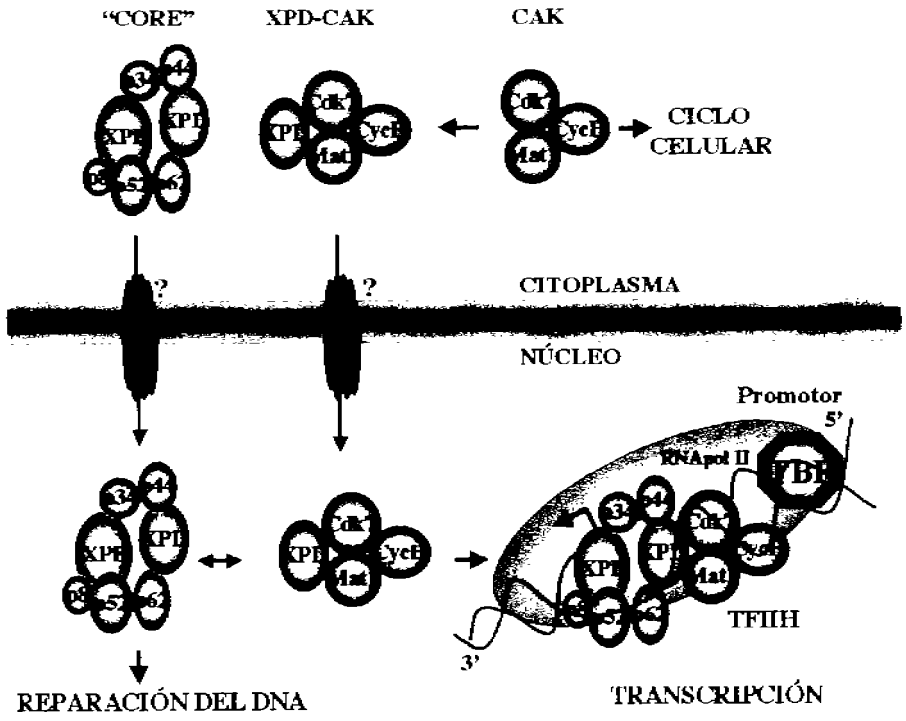
Se conoce poco sobre los mecanismos de importe nuclear de los componentes de la maquinaria basal de transcripción durante la activación de los genes cigóticos. Se sabe que la RNA pol II está presente en los núcleos del blastodermo sincicial entre los ciclos mitóticos 8 y 10, y que su CTD se hiperfosforila hasta el blastodermo celular de *Drosophila*. Así también, se reportó que la TBP, un componente de TFIID, se encuentra en los núcleos del ciclo mitótico 8 del blastodermo sincicial (Seydoux y Dunn, 1997; Wang y Lindquist, 1998; Leclerc et al., 2000). Hasta la fecha se desconoce si el ensamblaje de las subunidades de TFIID ocurre en el citoplasma o en el núcleo y si éste es necesario para su importe nuclear.

En el presente trabajo encontramos que el complejo de transición CAK-XPD está presente en el citoplasma de los embriones tempranos de *Drosophila* y no interacciona físicamente con XPB. Además, se presentan datos que apoyan la interacción entre XPB y XPD en el citoplasma, sin interactuar físicamente con el CAK. Se sabe que esta interacción se da solamente con la presencia de las demás subunidades del "core" de TFIIH (Schultz et al., 2000; Jawhari et al., 2002). Por lo tanto, proponemos que el ensamblaje del "core" es realizado en el citoplasma del blastodermo sincicial y celular de los embriones, y que éste no interacciona con el complejo transitorio CAK-XPD. También sugerimos que las subunidades del "core" y CAK se ensamblan en el núcleo para formar a TFIIH durante la activación de la transcripción cigótica (Figura 20a). Por lo tanto, proponemos que el ensamblaje del "core" en el citoplasma es necesario para su importe nuclear. Esto último es apoyado con el experimento de interacción entre la proteína XPB y un anticuerpo anti-XPB. En este experimento, encontramos que al neutralizar la proteína XPB en el citoplasma se interrumpe el importe nuclear de XPD y XPB. Este mismo fenómeno se observó cuando neutralizamos a la proteína XPD en el citoplasma del embrión de *Drosophila*. Algo similar ocurre en el importe nuclear de las subunidades de la RNA pol II. Lo que se observó fue que las subunidades Rpb4p y Rpb7p de la RNA pol II de *Saccharomyces cerevisiae* tienen un localización subcelular muy parecida, por lo que se propone la formación de un heterodímero en el citoplasma para un adecuado importe

nuclear de ambas proteínas. Lo anterior se sugirió porque se observó a las proteínas Rpb7p y Rpb4p retenidas en el citoplasma de células mutantes en los genes *Rpb4p* y *Rpb7p*, respectivamente. Datos similares se observaron cuando se evaluó la dinámica subcelular de ambas subunidades en un fondo mutante para una nucleoporina (*nup49*), la cual participa en el importe nuclear de ambas subunidades (Selitrennik et al., 2006). Así también, se observó que mutaciones en el N-terminal de la segunda subunidad más grande de la RNA pol III (Rpc128) afectan el importe nuclear no solamente de ella misma si no también de otras subunidades de la RNA pol III. Estos datos sugieren que las subunidades de la RNA pol III se importan al núcleo de manera coordinada, para lo cual podría requerirse un ensamblaje previo de los subcomplejos de la RNA pol III en el citoplasma (Hardeland y Hurt, 2006). Todo lo anterior es ejemplo del importe nuclear de complejos ensamblados en el citoplasma, pero poco se sabe de cómo es importado un complejo de proteínas a través del poro nuclear. Es decir, no se sabe si las subunidades son importadas de manera independiente o si el complejo de proteínas es importado como un todo. Parece ser que la importación de los complejos de proteínas se da mediante el paso de cada una de las subunidades en lugar de que todo el complejo pase ensamblado. Por ejemplo, a través del poro nuclear pasa cada proteína ribosomal en lugar de todo el ribosoma o solamente las histonas en lugar de todo el octámero de histonas (Rout et al., 1997; Mosammaparast et al., 2001; Mosammaparast et al., 2002). Pensamos que probablemente el importe nuclear del "core" de TFIIH utiliza el mecanismo de carga-importinaα-importinaβ. Debido a que se ha encontrado que las subunidades del "core" de TFIIH: XPB, p62 y p44, poseen una secuencia de aminoácidos básicos ("karyophilic proteins") que comúnmente se conoce como secuencia de localización nuclear (NLS; Chook y Blobel, 2001). En el importe nuclear, esta secuencia es reconocida por los componentes del mecanismos de carga-importinaα-importinaβ para translocar a las proteínas del citoplasma al núcleo (Anexo, Figura 1; Macara, 2001).

Por otra parte, observamos que la transcripción del gen *ftz* es afectada cuando se interrumpe el importe nuclear del "core". Por lo que sugerimos que el paso de los subcomplejos de TFIIH hacia al núcleo es esencial para el inicio de la transcripción de los genes cigóticos (Figura 20b).

a


"CORE"
XPD-CAK
CAK
CICLO CELULAR
CITOPLASMA
NÚCLEO
Promotor
5'
RNApol II
TFIIH
3'
REPARACIÓN DEL DNA
TRANSCRIPCIÓN

b

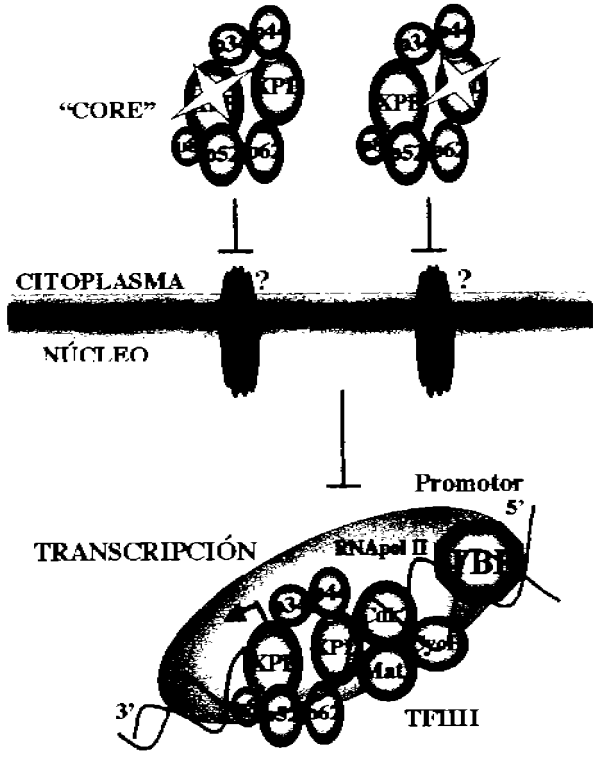

"CORE"
CITOPLASMA
NÚCLEO
Promotor
5'
TRANSCRIPCIÓN
RNApol II
TFIIH
3'

Figura 20. Modelo de la translocación nuclear y del ensamblaje de TFIIH en la activación de la transcripción de los embriones de la mosca. (a) Los subcomplejos "core" y CAK de TFIIH tienen dinámicas subcelulares diferentes en los embriones tempranos de *Drosophila*. Antes de la activación de la transcripción de los genes cigóticos, los componentes del "core" y CAK se encuentran preferencialmente en el citoplasma. En el inicio de la transcripción, el subcomplejo transitorio CAK-XPD es formado. En seguida, el subcomplejo transitorio CAK-XPD y el "core" se unen en el núcleo para formar al complejo TFIIH. Durante la transcripción, TFIIH, RNA pol II y los factores generales de la transcripción se anclan a los promotores de los genes cigóticos para sintetizar sus respectivos mRNA. (b) La presencia de las helicasas XPB y XPD en el "core" de TFIIH se necesita para el importe nuclear del "core". Ya que si se neutraliza a XPB o XPD se afecta la translocación del "core" hacia al núcleo. Así también, la transcripción de los genes cigóticos es afectada con la neutralización de ambas proteínas.

En resumen, la activación de la transcripción ocurren durante el desarrollo temprano de muchos organismos. Pero, la etapa del desarrollo en la cual se da la activación de la transcripción varia entre las diferentes especies. Se sabe que la activación de la transcripción se lleva acabo durante la transición de la blástula media en *Drosophila*, *Xenopus* y en el pez cebra (MBT; Newport y Kirschner, 1982; Edgar y Schubiger, 1986). Hasta la fecha no se sabe como se da la activación de la transcripción cigótica. Pero, en el presente trabajo se apoya la siguiente hipótesis: la deficiencia de la función de un activador transcripcional regula el inicio de la transcripción cigótica durante el desarrollo temprano del embrión (Almouzni1 y Wolffe, 1995). Esta ausencia de actividad puede ser debido a la fosforilación en el T-loop de CDK7 y en otras subunidades de TFIIH. Se sabe que este tipo de modificación postraduccional da como resultado una inhibición de la actividad de cinasa de TFIIH, seguido de una falta de actividad transcripcional durante la mitosis (Akoulitchev y Reinberg, 1998; Long et al., 1998). Por lo anterior y retomando nuestros resultados obtenidos de los experimentos de neutralización, sugerimos que el importe nuclear de los componentes de la maquinaria basal de la transcripción durante el blastodermo sincicial da como resultado la activación de la transcripción de los genes cigóticos, siendo esto esencial para su expresión. La RNA pol II, TBP y TFIIH son algunos de los componentes de la maquinaria basal de la transcripción que pudieran ser esenciales para el activación de la transcripción cigótica.

8. CONCLUSIONES.

- En los resultados obtenidos en las inmunoticiones de embriones tempranos de la mosca: observamos que el "core" está preferencialmente en el núcleo y el CAK está preferencialmente en el citoplasma en el momento de la activación de los genes cigóticos. Por lo que podemos decir que el "core" y el CAK de TFIIH tienen dinámicas subcelulares diferentes en la embriogénesis temprana de *Drosophila*.
- En los experimentos de inmuno-blot, realizados de fracciones citoplásmicas y nucleares obtenidas con dos métodos de centrifugación diferencial, pudimos observar que cuando no se emplea una solución con sacarosa, XPB ("core") está preferencialmente en el núcleo y CDK7 (CAK) se encuentra preferencialmente en el citoplasma. Pero, cuando se obtienen fracciones puras o enriquecidas del citoplasma y núcleos en centrifugaciones realizadas con una solución que contiene sacarosa, XPB y CDK7 están tanto en el núcleo como en el citoplasma. Lo anterior se debe a que los organelos o complejos multiproteicos que están en el citoplasma, como es el "core", flotan debido a que tienen una densidad menor a la que tiene la solución con sacarosa. Por otra parte, el CAK fue observado en el extracto nuclear porque la centrifugación de los núcleos en una solución con glicerol permitió separar los diferentes complejos multiproteicos nucleares con respecto a sus densidades. De esta manera, se obtuvieron diferentes fracciones nucleares solubles (SNF). Cada una de las fracciones nucleares por separado contienen diferentes complejos multiproteicos enriquecidos con diversas funciones en la transcripción. Por ejemplo, en una de las fracciones nucleares están los complejos multiproteicos transcripcionalmente activos mientras que en otra fracción nuclear se encuentran los complejos multiproteicos que inactivan la transcripción (Kamakaka, 1991; Kamakaka y Kadonaga, 1994).
- Los datos de inmunoticiones en las glándulas salivales de larvas del tercer estadio indican que el "core" y el CAK están preferencialmente localizados en el núcleo. Además, las preparaciones de cromosomas politénicos muestran que existen regiones cromosomales donde el "core" y el CAK de TFIIH co-localizan, pero hay regiones discretas en donde únicamente el CAK está localizado. Posiblemente, en estos lugares cromosomales solamente el CAK está participando en el "Capping" de los transcritos generados por la RNA pol II. Ya que se demostró que la inhibición química de CDK7

reduce la cantidad de los transcritos con "Cap", sin inhibir o alterar la transcripción mediada por la RNA pol II (Kanin et al., 2007).

- Los experimentos de ChIP demuestran que TFIIH está en los promotores de los genes *hb* e histona *H3*. Estos resultados nos indica que TFIIH participa en el inicio de la transcripción cigótica, ya que los genes evaluados son transcritos durante la activación de la transcripción del embrión de *Drosophila*.
- Los ensayos de CoIP indican que los subcomplejos "core" y CAK de TFIIH están presentes en el citoplasma, pero no están unidos formando a TFIIH. Este mismo ensayo muestra que en los núcleos transcripcionalmente activos, el "core" y CAK se unen para formar al complejo TFIIH.
- En los datos de neutralización de las subunidades de TFIIH se puede observar que el ensamblaje de XPB o XPD en el "core" de TFIIH se requiere para que el "core" entre al núcleo apropiadamente. Además, este experimento mostró que la neutralización de XPD y XPB no afectó el ciclo celular. En cambio, en la neutralización de CDK7, componente del CAK, se observó a todas las células arrestadas en mitosis y con aberraciones en los cromosomas mitóticos. También, la neutralización de XPB o XPD en el citoplasma indicó que alterando la localización nuclear del "core" es posible afectar la expresión del gen *fushi tarazu* en el inicio de la transcripción cigótica. Por lo anterior, podemos concluir que el importe nuclear del "core" de TFIIH es necesario para que se de una transcripción adecuada de los genes cigóticos durante la embriogenésis temprana de *Drosophila*.

9. PERSPECTIVAS.

Este trabajo abrió la posibilidad de plantear nuevas preguntas como por ejemplo:

- ¿Cuál o cuáles son los mecanismos de entrada de los subcomplejos de TFIIH del citoplasma al núcleo en los embriones temprano de *Drosophila*?
- ¿Por qué el CAK es preferencialmente citoplásmico en las células de los embriones, y por qué es preferencialmente nuclear en células de glándulas salivales?
- ¿Cuál es la función del CAK en las regiones cromosomales en donde solamente está él sin el "core"?.

Para abordar estas preguntas podemos plantear las siguientes perspectivas.

❖ Con la finalidad de identificar el mecanismo que regula la posición del CAK y del "core" en el citoplasma pretendemos hacer lo siguiente:

(1) Secuenciar a las proteínas de los CoIPs que se realizaron con los extractos citoplásmicos y los anticuerpos contra CDK7 y XPB. Esto con el fin de identificar factores que se asocien al CAK y "core".

(2) Tratar de afectar el tránsito del citoplasma al núcleo tanto del "core" como del CAK mediante el uso de inhibidores del citoesqueleto o del ciclo celular.

❖ Con el propósito de saber en que grupo de genes está posicionado mayoritariamente el CAK.

(1) Trataremos de mapear las bandas de los cromosomas politénicos en las cuales solamente se observa el CAK, e identificar que genes están en dichas regiones. Con esto podemos determinar en que promotores está el CAK sin el "core", y si éstos se están transcribiendo en las glándulas salivales.

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10.1. Páginas electrónicas.

1.http://www.mun.ca/biology/scarr/Fig25_05.gif

2.http://images.google.com.mx/imgres?imgurl=http://www.learner.org/channel/courses/biology/images/archive/fullsize/1985_fs.jpg&imgrefurl=http://www.learner.org/channel/courses/biology/archive/images/1985.html&h=198&w=350&sz=12&tbnid=cJsiccqsSJ7T2M:&tbnh=68&tbnw=120&hl=es&start=3&prev=/images%3Fq%3Dgap%2Bgenes%26gbv%3D1%26svnum%3D10%26hl%3Des%26sa%3DG

3.http://nobelprize.org/nobel_prizes/medicine/laureates/1995/illpres/l-fly-larva.gif

11. ANEXO.

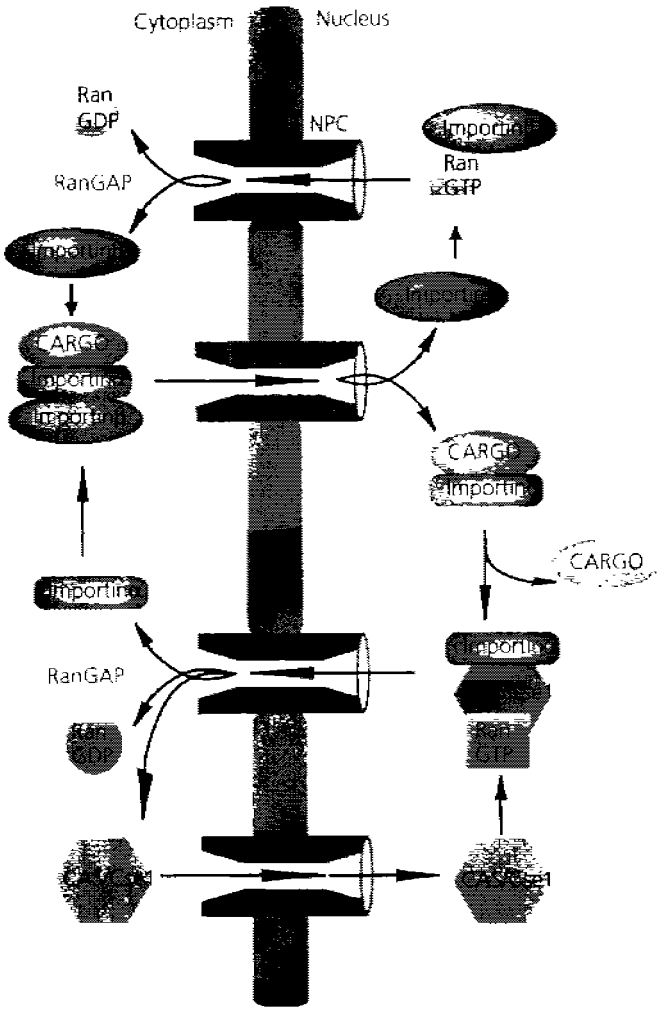

Figura 1. Mecanismo de importe nuclear carga-importinaα-importinaβ. En esta figura se muestra que la proteína con NLS (carga) a importar se une a la importinaα y en seguida la importinaβ se ensambla a este complejo. Una vez formado el complejo carga-importinaα-importinaβ en el citoplasma, éste se transporta al núcleo a través del poro nuclear (NPC). La importinaα requiere de la proteína acarreadora CAS/Cse1 para exportarse al citoplasma. A su vez, este complejo para exportarse al núcleo requiere de RanGTP (GTPasa pequeña que regula el transporte citoplásmico-nuclear, ya que se puede unir selectivamente a diferentes factores del transporte). Este compuesto ternario es separado en sus componentes en el citoplasma por hidrólisis de GTP (guanosín trifosfato). También, la importinaβ para translocarse al citoplasma requiere de la presencia de RanGTP pero no de la presencia de una proteína acarreadora. Esquema modificado de Macara, 2001.

12. GLOSARIO.

12.1. Abreviaturas y conceptos.

ATC	Activación transcripcional cigótica.
ATPasa	Enzima cuyo sustrato es el adenosín trifosfato.
atrx	Mutaciones en este gen causan *alpha thalassemia/mental retardation syndrome X-linked.*
Bicoid	Proteína organizadora del desarrollo de la parte anterior del embrión de *Drosophila.*
BSF	Factor de la estabilidad de Bicoid.
CAK(s)	Cinasa activadora de Cdks.
CAK-XPD	Complejo transitorio de TFIIH.
Cap	7-metilguanosina.
Capping	Etapa de maduración del mRNA.
Cdc2/Cdk1	Cinasa dependiente de ciclina que regula la entrada a mitosis.
CDK2	Cinasa 2 dependiente de ciclina que tiene funciones en la progresión del ciclo celular.
CDK7/Dmcdk7/hCDK7	Cinasa 7 dependiente de ciclina del CAK de TFIIH.
CDK8	Cinasa 8 dependiente ciclina.
CDK9	Cinasa 9 dependiente ciclina.
Cdks	Cinasas dependientes de ciclinas.
ChIP	Inmunoprecipitación de la cromatina.
Ciclina H1	Subunidad del CAK de TFIIH.
Ciclina	Proteína con función en la progresión de ciclo celular.
CoIP	Co-inmunoprecipitación de proteínas.
Core	Núcleo de TFIIH.
CTD	Carboxi-terminal de la subunidad grande de la RNA pol II.
Disco imagal o imaginal	Tejido de la larva que formará un órgano con una o más funciones en la mosca adulta.
DNA	Ácido desoxirribonucleico.

fushi tarazu (ftz)	Gen cigótico.
GFP	Proteína verde fluorescente.
GMP	Guanosín monofosfato.
GTP	Guanosín trifosfato.
H3	Gen de la histona H3.
heat shock protein (hsp70)	Gen que responde al choque térmico.
hunchback (hb)	Gen cigótico.
IP	Inmunoprecipitación de proteínas.
MAT1/hMAT1	Subunidad del CAK de TFIIH.
MBT	Blástula media.
mRNA	RNA mensajero.
NLS	Secuencia de localización nuclear.
p62	Subunidad del "core" de TFIIH.
p8/TTDA	Subunidad del "core" de TFIIH.
PCR	Reacción en cadena de la polimerasa.
REN	Mecanismo de reparación por escisión de nucleótidos.
REN-AT	REN acoplada a la transcripción.
REN-GG	REN general del genoma.
RNA pol	polimerasa de RNA.
RNA	Ácido ribonucleico.
Salivary gland secretion 5 (Sgs5)	Gen que se expresa en la glándula salival.
SC	Síndrome de Cockayne.
SOD1	Proteína superóxido dismutasa 1.
TAFs	Factores asociados a la proteína TBP.
TBP/hTBP	Proteína de unión a la caja TATA.
TFIIA	Factor de transcripción IIA.
TFIIB	Factor de transcripción IIB.
TFIID	Factor de transcripción IID.
TFIIE	Factor de transcripción IIE.
TFIIF	Factor de transcripción IIF.
TFIIH	Factor de transcripción IIH.

TFIIx	Factores multiproteicos de la transcripción mediada por la RNA pol II.
T-loop	Dominio de las cinasas dependientes de ciclinas.
TTD	Tricotiodistrofia.
TTDA/p8	Subunidad del "core" de TFIIH.
UV	Luz ultravioleta.
XP	Xeroderma pigmentosum.
XPB/Haywire (DmXPB)	Helicasa 3´-5´ del "core" de TFIIH.
XPD/DmXPD	Helicasa 5´-3´ del "core" de TFIIH.

12.2. Reactivos.

µg	Microgramo(s).
µl	Microlitro(s).
Cy2	Fluoróforo análogo a la fluoresceína.
Cy3	Fluoróforo análogo a la rodamina.
Deoxicolato de Sodio	Detergente que provoca la lisis de membranas celulares.
Fab	Fracción de un anticuerpo que identifica al antígeno.
FO	Formaldehído.
g/rcf	Campo gravitacional de la tierra/fuerza centrífuga relativa.
gr	Gramo(s).
hr(s)	Hora(s).
IGEPAL	Detergente no iónico.
Ioduro de propidio	Fluoróforo que se intercala en el DNA.
min	Minuto(s).
ml	Mililitro(s).
mM	Milimolar.
nM	Nanomolar.
O/N	12 hrs de incubación.
PBS	Solución amortiguadora de fosfato.
Proteinasa K	Proteasa.
RNAsa	Enzima que hidroliza al RNA.
SDS	Detergente iónico.
SDS-PAGE	Electroforesis en gel de poliacrilamida desnaturalizante.
SNF	Fracción nuclear soluble.
Sytox Green	Fluoróforo que se intercala en el DNA.
TA	Temperatura ambiente.
TBS	Solución amortiguadora de Tris-base.
TE	Solución de Tris-HCl.

Tritón X-100	Detergente no iónico.

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TFIIH trafficking and its nuclear assembly during early *Drosophila* embryo development

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Summary

We present the first analysis of the dynamics of the transcription DNA-repair factor TFIIH at the onset of transcription in early *Drosophila* development. TFIIH is composed of ten polypeptides that are part of two complexes – the core and the CAK. We found that the TFIIH core is initially located in the cytoplasm of syncytial blastoderm embryos, and that after mitotic division ten and until the cellular blastoderm stage, the core moves from the cytoplasm to the nucleus. By contrast, the CAK complex is mostly cytoplasmic during cellularization and during gastrulation. However, both components are positioned at promoters of genes that are activated at transcription onset. Later in development, the CAK complex becomes mostly nuclear and co-localizes in most chromosomal regions with the TFIIH core, but not in all sites, suggesting that the CAK complex could have a TFIIH-independent role in transcription of some loci. We also demonstrate that even though the CAK and the core coexist in the early embryo cytoplasm, they do not interact until they are in the nucleus and suggest that the complete assembly of the ten subunits of TFIIH occurs in the nucleus at the mid-blastula transition. In addition, we present evidence that suggests that DNA helicase subunits XPB and XPD are assembled in the core when they are transported into the nucleus and are required for the onset of transcription.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/119/18/3866/DC1

Key words: TFIIH, *Drosophila*, Early embryo, Dynamics

Introduction

In eukaryotic cells, RNA polymerase II (RNA pol II)-mediated transcription requires several general transcription factors and chromatin-remodelling complexes. TFIIH is one of the general transcription factors that have been extensively characterized. TFIIH is composed of ten subunits that can be subdivided in two complexes. The core complex contains six proteins, namely p8, p34, p44, p52, p62 and XPB; whereas the Cdk-activating kinase complex (CAK) includes CDK7, Cyclin H and MAT1 (Giglia-Mari et al., 2004). XPD is component of both complexes and it seems to be the bridge between the core and the CAK (Egly, 2001). TFIIH has several enzymatic activities: XPB and XPD are DNA helicases, CDK7 is a kinase and the p44 subunit is a ubiquitin ligase (Takagi et al., 2005). Mutations in either of the genes encoding XPB or XPD can produce three hereditary human disorders known as Xeroderma pigmentosum (XP), patients with combined symptoms of Xeroderma pigmentosum and Cockayne syndrome (XP/CS) and Trichothiodystrophy (TTD) (Lehmann, 1998; de Boer and Hoeijmakers, 1999; Nance and Berry, 1992; Cleaver, 2000; Rolig and McKinnon, 2000; Lehmann, 2002). It has recently been reported that mutations in the new TFIIH subunit p8 also produce TTD (Giglia-Mari et al., 2004).

Besides its dual role in transcription, TFIIH also has a central role in the mechanism of nucleotide-excision repair (NER) (Egly, 2001; Zurita and Merino, 2003), and there is increasing evidence that the CAK complex is involved in cell-cycle regulation (Chen et al., 2003; Fisher, 2005). In transcription, TFIIH promotes the formation of the open complex and it is generally accepted that its kinase activity targets the CTD domain of the RNA pol II, in particular the phosphorylation of Ser5 in the heptapeptide repeat (Lu et al., 1992; Rossignol et al., 1997). These events facilitate the escape of the RNA pol II from the promoter. Interestingly, in vitro experiments have shown that in some promoters RNA pol II transcription can be achieved without TFIIH kinase activity (Mäkelä et al., 1995). Another report shows that the conditional ablation of the MAT1 subunit in Schwann cells does not affect transcription (Korsisaari et al., 2002). By contrast, CDK7 is required for the transcription of the *Drosophila* heat shock genes and RNA pol II phosphorylation in third-instar larvae salivary glands (Schwartz et al., 2003). Also, the development of structures that require high levels of transcription are affected in a *Cdk7* mutant (Merino et al., 2002).

In *Drosophila*, a *Cdk7* dominant-negative mutant was found to delay embryonic transcription (Leclerc et al., 2000). In addition, CDK7 is required for both mRNA transcription and cell-cycle progression in early *Caenorhabditis elegans* embryos (Wallenfang and Seydoux, 2002). Therefore, the role of the CAK complex during development is still not well understood.

In many animals, the early embryo does not require the synthesis of RNA. Activation of transcription generally occurs at the mid-blastula transition and is an essential requirement for subsequent developmental stages (Davidson, 1986). In *Drosophila*, early embryo development passes through a

syncytial stage, in which 13 synchronic nuclear divisions occur without cell division. In addition, early mitotic divisions occur with partial nuclear envelope breakdown (Stafstrom and Staehelin, 1984). No transcription occurs during these synchronic nuclear divisions, with the exception of the cycle elongation in division 10, in which *histone*, *Gap* and *pair rule* genes begin to be transcribed. During cellularization, global transcription is activated and there is an increase in phosphorylation of the RNA pol II C-terminal domain (CTD) (Seydoux and Dunn, 1997). Therefore, early fly embryo development is an excellent model to analyze the intracellular traffic and dynamics of the basal transcription machinery at transcription onset at the mid-blastula transition (MBT).

In this work, we analyzed the intracellular dynamics of the TFIIH core and CAK complexes in the early *Drosophila* embryo at the MBT. Our results demonstrate that most of the CAK and the core complexes follow different dynamics and subcellular distribution. However, both complexes seem to be involved in gene activation at the onset of transcription. We also demonstrate that the translocation of XPB and XPD into the nucleus at this stage is inhibited by the inactivation of any subunit of the TFIIH core and that this affects transcription activation. These results are the first to address the assembly and trafficking of a transcriptional complex at the onset of zygotic transcription at MBT.

Results

Differential dynamics of the subcellular distribution of the core and CAK complexes in the embryonic development of *Drosophila*

To determine the dynamics of the core and CAK complexes in the early *Drosophila* embryo, we used polyclonal antibodies that recognize two components of the core (XPD and XPB) and two of the CAK complex (CDK7 and MAT1). Embryos were immunostained for the core and the CAK components and visualized on a confocal microscope. Fig. 1 shows XPB (*haywire* in *Drosophila*) (Mounkes et al., 1992) distribution at different stages of embryo development. XPB protein is initially deposited in the early syncytial blastoderm by maternal contribution and is located in the cytoplasm (Fig. 1a). By nuclear division eight, nuclei migrate to the embryo periphery while XPB also concentrates in the embryo border region while remaining cytoplasmic (Fig. 1b). Interestingly, at this stage XPB surrounds the nuclei in cytoplasmic domains or energids that delimitate the space that each cell will occupy in the cellular blastoderm (Fig. 1c,c'). By nuclear division ten, part of the XPB signal is located inside the nuclei at the embryo periphery (Fig. 1d,g). At this stage syncytial nuclei have a prolongation of their replication phase that allows the transcription of the first zygotic genes. By cellularization at nuclear division 13-14, most XPB signal is located inside the nucleus (Fig. 1e,h). At this stage RNA pol II transcription is activated in the early *Drosophila* embryo. During gastrulation, XPB is preferentially maintained inside the interphase nuclei while it is clearly excluded from mitotic chromosomes (Fig. 1f,i). XPD displays identical dynamics to XPB, and both proteins colocalize throughout development (supplementary material Figs S1, S2).

Interestingly, we found that at the same developmental stages, CDK7 distribution is different. CDK7 is also deposited in the syncytial blastoderm embryo by maternal contribution and it is located homogeneously in the cytoplasm (Fig. 2a). Contrary to the observed dynamics of XPB and XPD at nuclear division 10, CDK7 is preferentially detected in the cytoplasm (Fig. 2b). In more advanced stages, such as cellular blastoderm and gastrulation, most of the CDK7 signal is still cytoplasmic (Fig. 2c,d). An identical pattern was observed using a MAT1 antibody (Fig. 2e-h), showing that both CAK components have

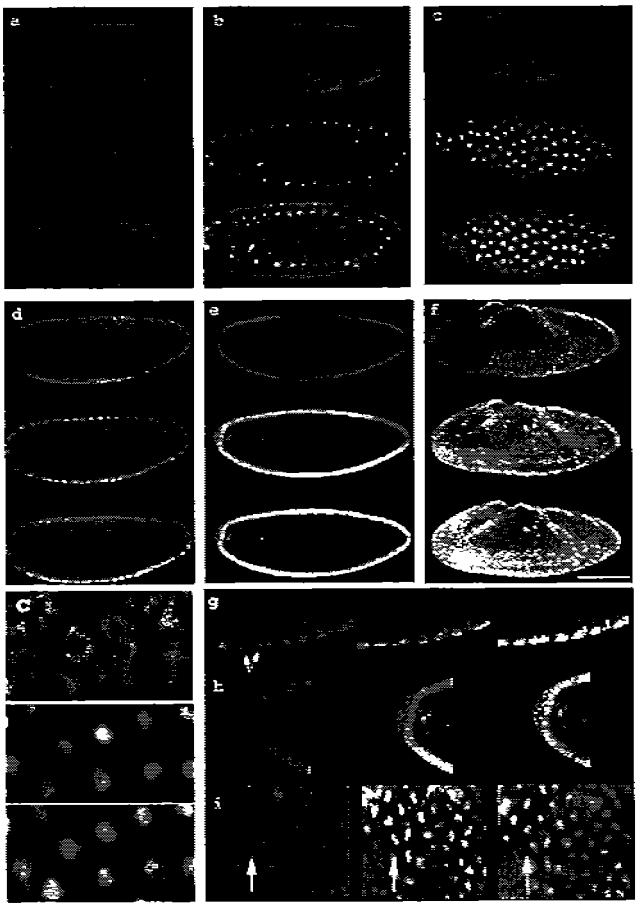

Fig. 1. XPB subcellular distribution in early *Drosophila* embryogenesis. XPB was localized using a polyclonal antibody. XPB signal is in red; DNA is in green after Sytox green staining. (a) Syncytial blastoderm embryo after the first nuclear division (two nuclei, stage 2). (b) Syncytial blastoderm embryo at stage 8; the nucleus starts to move towards the embryo periphery and XPB remains cytoplasmic. (c,c') A similar embryo at stage 8 of development, but only the surface staining shown. Note that XPB surrounds the nucleus in cytoplasmic domains or energids. (d) Syncytial blastoderm embryo at stage 10. XPB enters into the nuclei, which are now located in the embryo periphery. (e) Cellular blastoderm embryo. At this stage, most of XPB signal is located inside the nucleus. (f) Gastrulated embryo. XPB is preferentially nuclear in the interphase nuclei, but excluded from mitotic chromosomes. (g) Amplification of the periphery of a stage 10 embryo. The arrows indicate XPB signal inside the nucleus. (h) Amplification of a cellular blastoderm embryo. (i) Amplification of a gastrulated embryo. The arrow indicates a group of mitotic chromosomes. Bar, 100 μm.

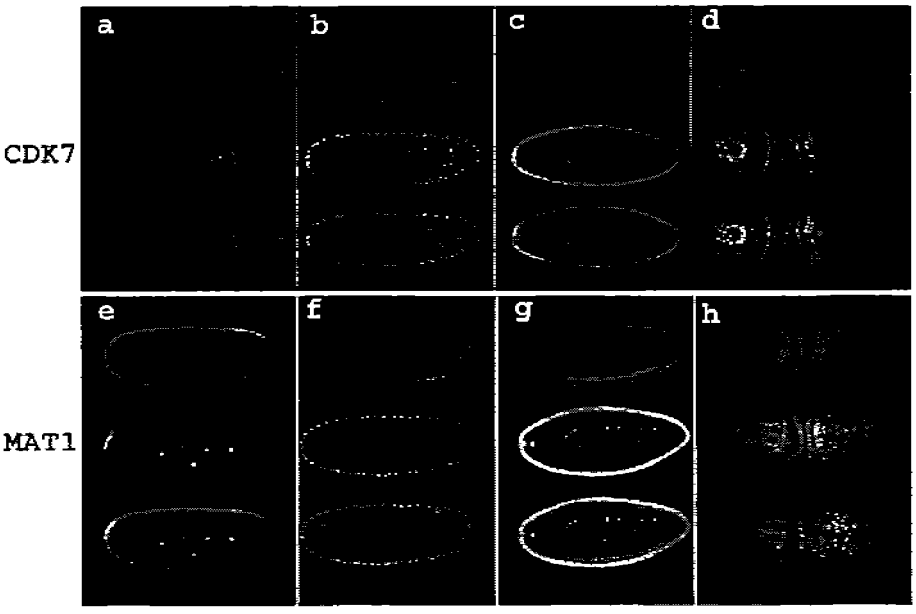

Fig. 2. CDK7 and MAT1 subcellular distribution in early *Drosophila* embryogenesis. Cellular distribution of CDK7 (red signal) at different embryo developmental stages from syncytial blastoderm to gastrulation (a-d). DNA is shown in green. Note that the CDK7 signal is preferentially located in the cytoplasm at all stages. Distribution of the CAK complex protein MAT1 in early *Drosophila* development (e-h). The red signal is MAT1 after immunostaining with a polyclonal antibody (see Materials and Methods) and the green signal is DNA. (a,e) Early syncytial blastoderm embryos. (b,f) Syncytial blastoderm embryos at nuclear division 10. (c,g) Cellular blastoderm embryos. (d,h) Embryo during gastrulation.

similar dynamics during embryonic development. From these results we conclude that the core and CAK complexes of TFIIH have different behaviours during early fly embryo development.

Since it has been reported that XPD anchors the CAK complex with TFIIH, we performed simultaneous immunostaining with XPD and CDK7. At the resolution of the confocal microscope, the two proteins appear not to colocalize (Fig. 3). Western blot experiments with cytoplasmic and nuclear fractions from cellularized embryos confirmed that most of the XPD signal is nuclear and that CDK7 is preferentially located in the cytoplasm (supplementary material Figs S1, S2). These data suggest that most of the core and CAK complexes do not interact during early *Drosophila* embryo development.

The CAK and the core complexes of TFIIH co-localize along most of the polytene chromosomes, but not in all sites

Our data show that most of the CAK and core complexes do not have the same cellular distribution in the early *Drosophila* embryo. However, it has been reported that CDK7 is necessary for correct HSP70 gene expression and it co-localizes with the active RNA pol II in polytene chromosomes (Schwartz et al., 2003). Therefore, we decided to analyze the subcellular distribution of the two TFIIH subcomplexes in

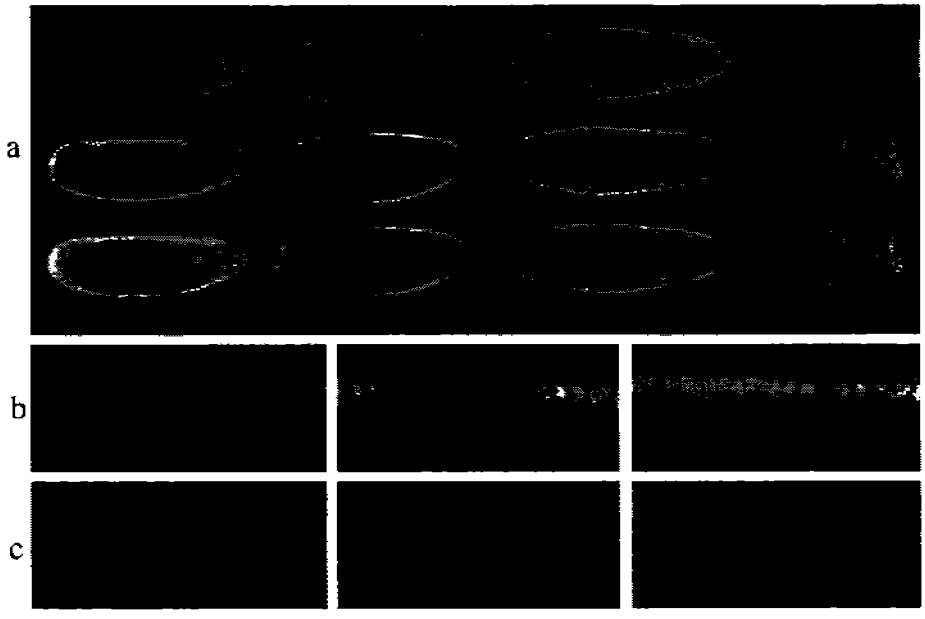

Fig. 3. Co-immunostaining of CDK7 and XPD during early fly embryogenesis from syncytial blastoderm to gastrulation. (a) XPD (green signal) is preferentially nuclear after mitosis number 10. CDK7 (red signal) is preferentially located in the cytoplasm at these developmental stages. (b) Amplification of a cellular blastoderm co-stained with CDK7 (red) and XPD (green). (c) Amplification of a gastrulated embryo co-stained with CDK7 (red) and XPD (green).

terminally differentiated tissues such as third-instar salivary glands. We found that in differentiated tissues, both the CAK (CDK7 and MAT1) and the core (XPD and XPB) are preferentially nuclear (Fig. 4a,b; data not shown). In polytene chromosome preparations, CDK7 and XPB co-localize at most chromosomal sites (Fig. 4c). However, we identified some regions where only CDK7, but not XPB or XPD is detectable (Fig. 4c enlarged image). Similar results were observed with chromosome immunostaining against MAT1 and XPD (Fig. 4d). These results show that, in contrast to the CAK cellular distribution in the *Drosophila* embryo, at larval stages this complex is preferentially nuclear and it co-localizes in most, but not all chromosomal sites with the TFIIH core subunits.

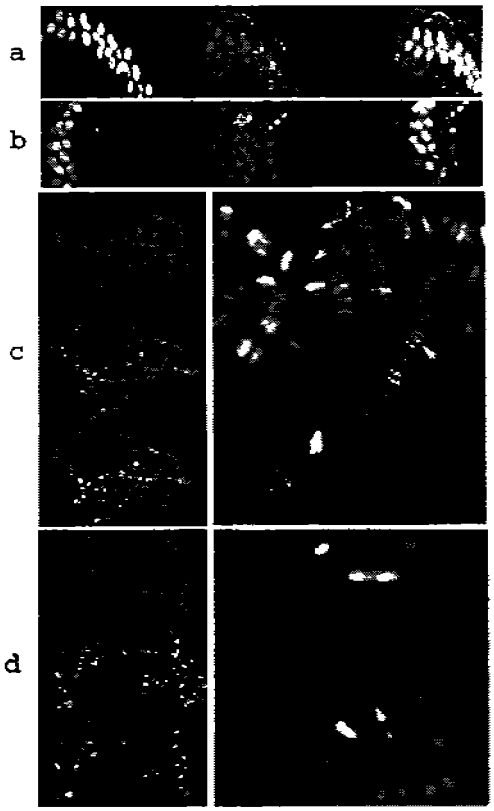

Fig. 4. Subcellular and chromosomal distribution of the CAK and the core complexes of TFIIH in third-instar larvae salivary gland and polytene chromosomes. (a) Immunolocalization of CDK7 shown in red and DNA staining in green. (b) Immunolocalization of XPD (red) and DNA (green) in third-instar salivary glands. (c) Co-immunostaining of CDK7 (red) and XPD (green) in polytene chromosomes. Most of the two proteins colocalize in a specific euchromatin banding pattern and the signal is enriched in the puffs. An amplified view of the CDK7/XPD immunolocalization in polythene chromosomes is shown. The arrows indicate sites where CDK7 does not colocalize with XPD in the chromatin. (d) Chromosomal distribution of MAT1 and XPD in polytene chromosomes. The red signal is MAT 1 and the green signal is XPD. The arrows indicate an example of a site where MAT1 is localized without XPD in an amplified image.

The CAK and the core subcomplexes of TFIIH are positioned in gene promoters activated at the onset of transcription in the early *Drosophila* embryo

The immunolocalization experiments in early *Drosophila* embryos suggest that most of the CAK is in the cytoplasm and that it may not be involved in transcription or that it has a limiting role at these stages. However, confocal observations and western blots against different cellular fractions may not be sensitive enough to distinguish a small amount of CAK that might be localized on the chromatin. On the other hand, it has been reported that the overexpression of a dominant-negative form of *cdk7* delays *fushi taratzu* (*ftz*) gene transcription (Leclerc et al., 2000). Although the authors argue that this is a clear transcriptional defect, a problem in the cell cycle cannot be ruled out. Defects in the cell cycle caused by *Cdk7* mutations may affect the transition from synchronic nuclear division to asynchronous divisions and therefore delay the onset of transcription. Consequently, we decided to explore whether CAK participates in the transcription of genes that are activated between nuclear divisions 10-14. To achieve this, we performed chromatin immunoprecipitations (ChIP) experiments using antibodies against CDK7, XPD, XPB and RNA pol II. The selected targets were zygotic *hunchback* (*hb*) and *histone* H3 promoters. The zygotic *hb* and the H3 promoters are activated at nuclear division 10 elongation. As controls, we tested the promoter of the salivary-gland-specific gene *Sgs5*, which is not expressed in the embryo, the second exon of the *hb* gene and an exon of the *Drosophila ATRX* homologue, which is not expressed at these embryonic stages (our unpublished results).

ChIP experiments show that CDK7, XPD, XPB and RNA pol II are positioned in *hb*, and H3 promoters (Fig. 5). As expected, RNA pol II, but not CDK7, XPB and XPD, can be found at the *hb* exon. In addition, XPB, XPD and CDK7 are absent from the *Sgs5* promoter. In the ATRX exon none of these proteins were identified. These results show that even if CAK is not visualized by immunostaining the nuclei of early *Drosophila* embryos and that most of it is in the cytoplasm, some CAK is positioned at the promoter of genes that are being transcribed at these developmental stages, supporting a role of CAK in early *Drosophila* embryo transcription.

Association of the core and the CAK occurs in the nucleus at the onset of transcription

ChIP experiments indicate that the CAK and the core complexes are positioned in promoters that are active at the onset of transcription. On the other hand, confocal microscopy shows that in syncytial blastoderm as well as in cellular blastoderm stages, components of both complexes co-exist in the cytoplasm, but they do not necessarily interact.

To determine if the CAK and the core complexes subunits interact in the early fly embryo cytoplasm, we performed co-immunoprecipitations (CoIP) of 1-2.5 hour embryonic cellular and nuclear fractions. Anti-CDK7 and anti-XPB antibodies were used for CoIP experiments. Precipitated samples were analyzed for the presence of CDK7, MAT1, XPB and XPD by western blotting. After CoIP of the cytoplasmic fraction with anti-CDK7 antibody, MAT1, XPD and CDK7 can be detected in a western blot, forming the XPD-CAK transitory complex with CycH (Fig. 6). XPB does not co-immunoprecipitate with CDK7 (Fig. 6), suggesting that even though all the components

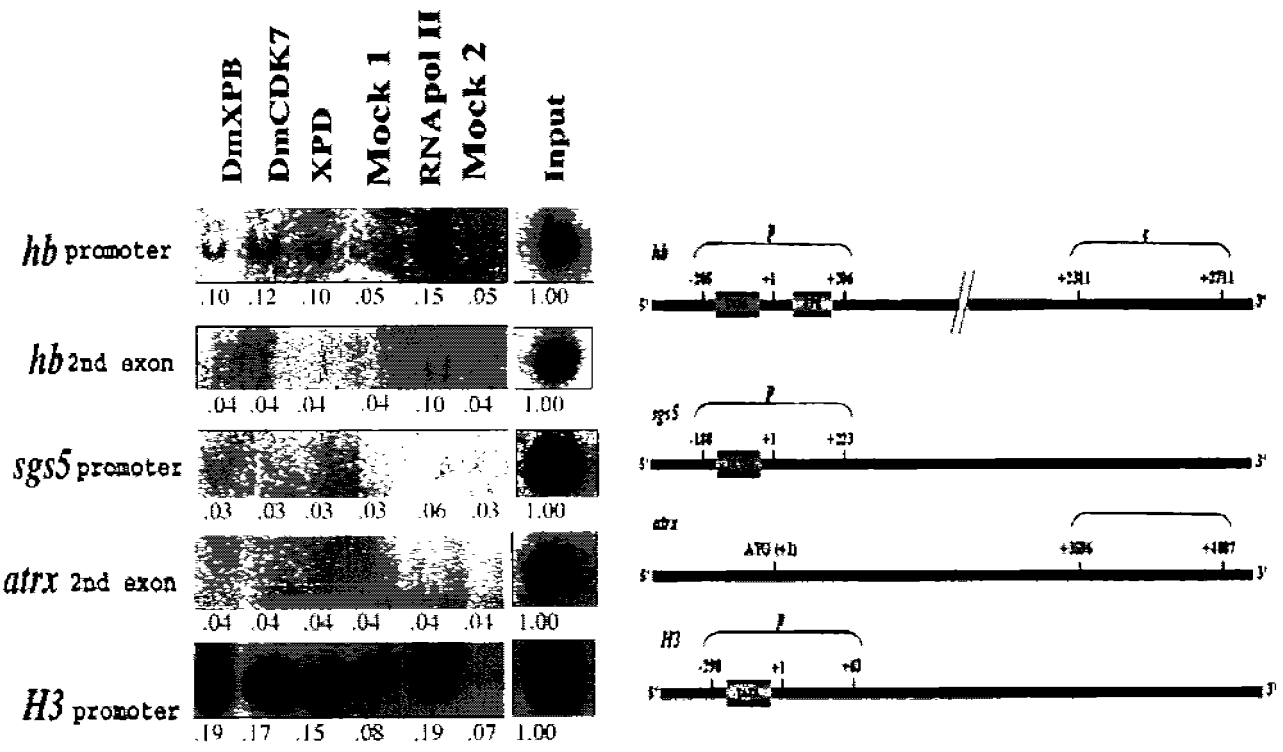

Fig. 5. CDK7, XPB and XPD are positioned at active promoters at the onset of transcription in early *Drosophila* embryos. Chromatin of embryos at 30-180 minutes of development was precipitated using antisera against CDK7, XPD, XPB and RNA pol II. Two neutral, unrelated IgG (Mock 1) and IgM (Mock 2) antisera were used as controls. The immunoprecipitated regions were amplified by PCR using oligonucleotides that cover the zygotic *hb* promoter, *hb* second exon, the *sgs5* promoter, the *atrx* second exon and the *H3* promoter; each amplified region is indicated in the figure. Input amplification is also shown for each PCR. The fraction of the input for each ChIP is indicated below each band. The sequences of the different promoters analyzed in this work are derived form the *Drosophila* core promoter database (http://www-biology.ucsd.edu/labs/Kadonaga/DCPD.html).

of TFIIH are present in the early *Drosophila* embryo cytoplasm, the complete assembled TFIIH, with all its ten subunits, is not present in this cellular fraction. On the other hand, in CoIP experiments of the cytoplasmic fraction using the anti-XPB antibody, XPD can be identified in western blots of CoIP material, but not CDK7 and MAT1 (Fig. 6). Complementary experiments with the CDK7 antibody using a nuclear fraction enriched with transcriptionally active chromatin (Kamakaka and Kadonaga, 1994), we found that CDK7, MAT1, XPD and XPB can be identified, suggesting that the CAK and the core are assembled in the nucleus thus forming the TFIIH ten-subunit factor (Fig. 6). CoIP of this fraction with the anti-XPB antibody pulls down XPD together with trace amounts of CDK7 and MAT1 (Fig. 6), indicating that most of the core complex is not interacting with the CAK, in agreement with the confocal observations.

XPB and XPD are assembled in the core of TFIIH to entry into the nucleus at the mid-blastula transition and are required for the onset of transcription

CoIP experiments with nuclear and cytoplasmic fractions from early embryos show that the CAK complex interacts with XPD in the cytoplasm, forming a transitory complex, and that this complex is probably transported inside the nucleus at the onset of transcription. These data also suggest that the core is assembled in the cytoplasm and transported to the nuclei. To determine if the core has to be assembled in the cytoplasm to enter the nuclei at transcription activation, we neutralized either XPB or XPD proteins in syncytial blastoderm embryos by injecting the corresponding antibodies. After injection, we looked for the localization of these two core components in cellularized and gastrulated embryos. We microinjected embryos 0-30 minutes old with the antibodies, fixed them, allowed to develop for 2 hours and stained them with either anti-XPB (in embryos injected with the XPD antibody), or anti-XPD (in embryos injected with the XPB antibody). The rationale for this experiment is that if both proteins are required to form the core to enter the nucleus, the neutralization of either one will interfere with the traffic of the other from the cytoplasm into the nucleus. As a control, we microinjected a GFP antibody, which does not recognize any protein in *Drosophila*. We also injected an antibody against CDK7 and stained for XPB and XPD. As control for an independent nuclear factor, we also stained the injected embryos with an anti-TBP antibody.

The results presented in Fig. 7 show that the neutralization of the XPD protein reduces the quantity of XPB inside of the nucleus, which remains preferentially cytoplasmic in embryos

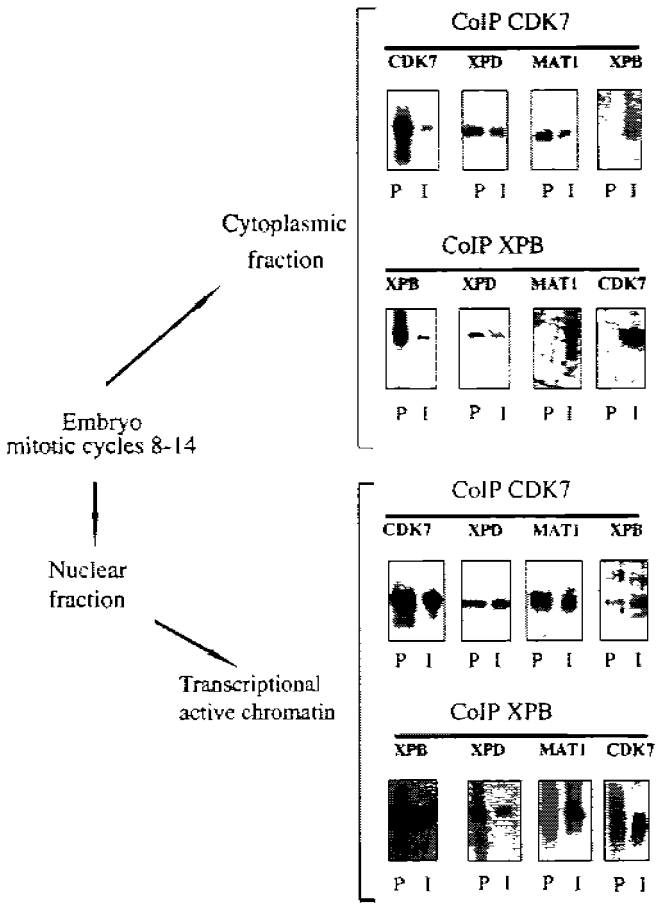

Fig. 6. CDK7 and XPB co-immunoprecipitations (CoIP) of cytoplasmic and nuclear fractions from embryos at mitotic stages 8-14. The cytoplasmic fraction was immunoprecipitated with either an anti-CDK7 or anti-XPB antibody. The nuclear fraction was co-immunoprecipitated with the anti-CDK7 antibody. The precipitated material and total proteins from cytoplasmic fraction before CoIP were analyzed by SDS-PAGE. The proteins were transferred to a membrane and analyzed by western blot experiments for the presence of CDK7, MAT1, XPB and XPD using specific antibodies as indicated. The same blot was reused for each antibody. The antibody used for CoIP is indicated in the figure. Lanes labeled P show the CoIP material and those labeled I show the input from the original cytoplasmic or nuclear fraction, equal amounts of protein were loaded in each lane.

initiating gastrulation, a stage in which we have demonstrated that XPB and XPD are preferentially nuclear (Fig. 7). When we injected the XPB antibody, XPD preferentially remains cytoplasmic (Fig. 7). On the other hand the control antibody did not affect the nuclear localization of either XPB or XPD in cellular blastoderm and gastrulated embryos (Fig. 7). Neither XPB nor XPD antibodies affect TBP nuclear localization, indicating that the effect is specific for TFIIH (Fig. 7). We were not able to visualize what happened when we neutralized CDK7, because the antibody immediately arrested development, producing aberrant mitosis, suggesting a role for CDK7 in the regulation of the first mitotic cycles of the early *Drosophila* embryo (Fig. 7). This observation disagrees with the previous report by Leclerc et al. (Leclerc et al., 2000) in which the expression of a *Cdk7* dominant-negative mutant of Cdk7 does not alter the timing of the first 13 embryonic nuclear cycles. However, differences in our work may be due to the use of different strategies and conditional *cdk7* mutant versus inactivation of Cdk7 by antibodies.

To determine if the inactivation of XPB or XPD affects transcription at the mid-blastula transition, we analyzed the expression of the *fushi taratzu* (*ftz*) gene by in situ hybridization in embryos that were microinjected with the XPB or XPD antibodies. In the early fly embryo, *ftz* is one of the first zygotic transcripts to be expressed and we found that the *ftz* expression pattern was affected in cellularized and gastrulated embryos injected with XPB and XPD antibodies (Fig. 8). These results show that XPB and XPD and are required for the onset of transcription at the mid-blastula transition.

Discussion

The CAK and the core complexes have different cellular distributions during fly development

Components of the TFIIH factor have a central role in three fundamental cellular functions: DNA repair, transcription and the control of the cell cycle. This implies that the TFIIH components interact with a diverse array of accessory factors. These interactions may occur in both the nucleus and the cytoplasm. Therefore, it is not surprising that different TFIIH subunits have different cellular distributions that depend on cell type and the stage of development. The results presented in this work show that most of the CAK complex remains in the cytoplasm during early embryo development and becomes preferentially nuclear in terminally differentiated tissues such as the larval salivary glands. By contrast, our results also show that the core complex is preferentially nuclear at the onset of transcription in the early embryo. Even though, most of the CAK complex is cytoplasmic in these embryonic stages, a fraction of this complex, together with the core, is positioned at promoters that are actively transcribed. These data suggest that the complete TFIIH factor participates at transcription onset at mid-blastula transition in the *Drosophila* embryo. The bulk of the CAK complex that remains in the cytoplasm probably participates in cell-cycle control.

The CAK complex phosphorylates Cdks that are fundamental for cell-cycle progression (Morgan, 1995). It has been reported that the activation of the Cdc2/Cyclin A and Cdc2/Cyclin B complexes in *Drosophila* requires Cdk7 (Larochelle et al., 1998; Larochelle et al., 2001). Cyclin A and B, but not Cdc2, levels fluctuate during these developmental stages (Foe et al., 1993). As a large proportion of Cdc2 is in the cytoplasm, it is possible that the main function of CAK in the cytoplasm may be the regulation of Cdc2 activity. In relation to this, it has been recently proposed that XPD levels regulate CDK7 activity and are therefore central for the control of the cell cycle in the *Drosophila* embryo. In accordance with this, it has been reported that levels of XPD but not other TFIIH core components like XPB, drop during mitosis, suggesting a role for CDK7 in regulating the cell cycle (Chen et al., 2003). However, we observed that XPB and XPD have a similar expression pattern and co-localize at all the analyzed stages, and that their relative levels do not change during mitosis

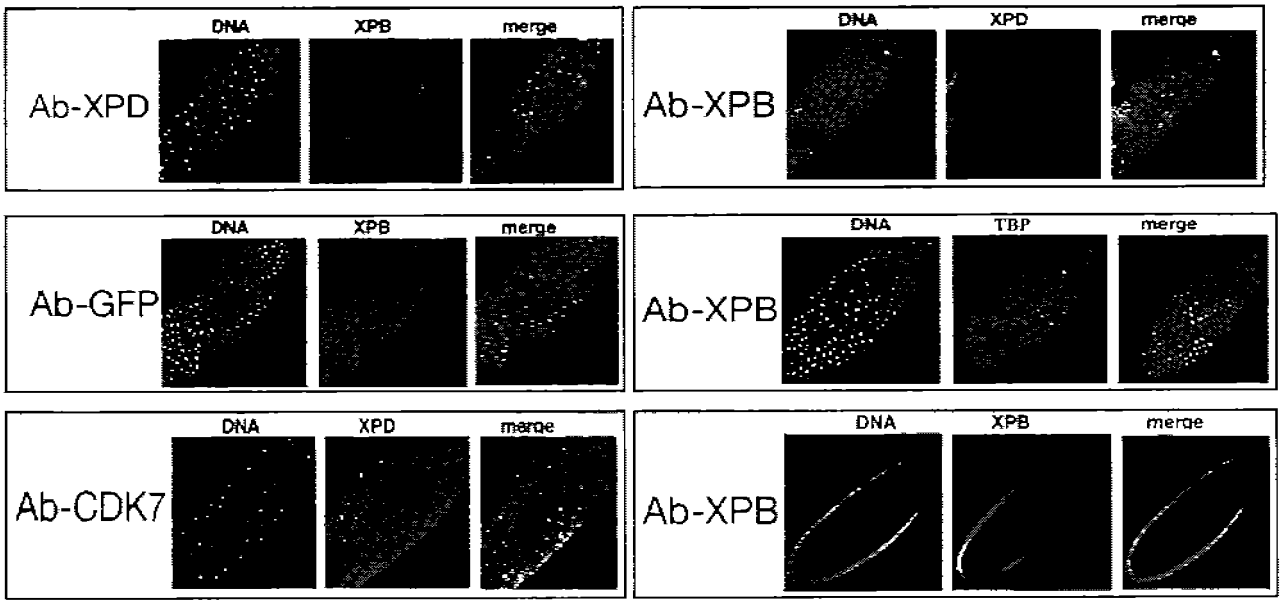

Fig. 7. XPB and XPD need to be assembled in the core of TFIIH for nuclear entry at the onset of transcription. Embryos at 0-30 minutes of development were injected with antibodies that specifically recognize XPB, XPD, GFP and CDK7. After injection, the embryos were allowed to develop for 2 hours, then were fixed and immunostained against either XPB or XPD as well as against TBP. The specific injected antibodies are indicated at the left of each panel. DNA staining with Sytox Green as well as the antibodies used for immunostaining are indicated at the top of each panel. Note that in the first panel (Ab-XPD), one half of the gastrulated embryo XPB is nuclear and in the other half is cytoplasmic. This effect is possible due to a gradient of neutralization of XPD by the antibody. Also note that the localization of XPD is cytoplasmic in a gastrulated embryo injected with the XPB antibody (Ab-XPB, middle panel). The injection of GFP antibody does not have any effect on the correct localization of XPB in a gastrulated embryo. Note that neither XPB nor XPD antibodies affect the TBP nuclear localization. Injection of the CDK7 antibody (Ab-CDK7 panel) arrests mitotic division and aberrant mitotic chromosomes are observed. The bottom right panel (Ab-XPB), shows a cellular blastoderm embryo stained with XPB after injection of its antibody. Note that a gradient of the nuclear XPB is observed in one half of the embryo and no signal is detected in the other half owing to the neutralization of XPB.

(supplementary material Figs S1, S2). In agreement with reports that show a major function of CDK7 in the M phase of the cell cycle (Larochelle et al., 1998), the inactivation experiments suggest a central role of CDK7 in the control of mitosis in the early blastoderm embryo (Fig. 7). In addition, the XPD antibody inactivation did not affect cell-cycle progression at these stages. On the other hand, we cannot discard a possible function of the CAK and core complexes in the cytoplasm beyond cell-cycle regulation. For instance, it is known that CAK also interacts with RNA, in particular CycH with the U1 snRNA and there are reports of that suggest a possible role of XPB in mRNA translation regulation in yeast (Kwek et al., 2002; Guylas and Donahoue, 1992).

In terminally differentiated tissues, the CAK and the core complexes become mostly nuclear. Why does this change in the subcellular distribution of the CAK complex occur? It is possible that the high levels of transcription that occur in the salivary glands cells require that most of CAK be preferentially nuclear and the regulation of other Cdks by the CAK complex may not be required.

In polytene chromosomes, the CAK and core complexes colocalize in most chromatin domains and are enriched in the puffs. This is to be expected because the two complexes form TFIIH, which is a component of the basal transcription machinery. However, there are some chromosomal regions where only the CAK complex is present, a surprising observation because its role in transcription has always been linked with TFIIH. This suggests that some loci may require the presence of CAK but not the other components of TFIIH to be transcribed. Alternatively, it is possible that CAK may be involved in functions other than the phosphorylation of the CTD domain of RNA pol II in the chromatin. Furthermore the chromosomal positions where CDK7 or MAT1 are located without the TFIIH core are the same in different chromosomal squashes, suggesting a specific unknown function. The future identification and characterization of the loci within these chromosomal positions will be useful to elucidate the role of CAK in the absence of TFIIH in the chromosomes.

The CAK and core complexes are translocated from the cytoplasm to the nucleus at the mid-blastula transition

Little is known about the transit of the basal transcription machinery from the cytoplasm to the nucleus at the onset of transcription in the early animal embryo. In the case of *Drosophila* it has been reported that RNA pol II is present in syncytial blastoderm nuclei between mitotic cycles eight to ten, and that significant phosphorylation of the CTD occurs at cellular blastoderm (Seydoux and Dunn, 1997). It has also been reported that the TATA-binding protein (TBP), a component of TFIID, enters the nuclei at mitotic division eight (Wang and Lindquist, 1998). Both RNA pol II and TFIID are composed of several subunits and it is not known if the assembly of these

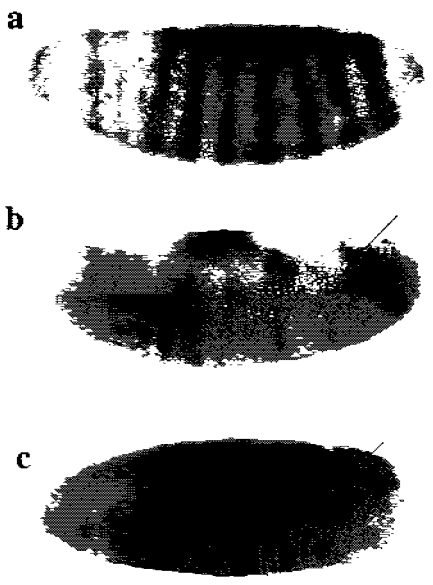

Fig. 8. XPB and XPD inactivation affects expression of the *ftz* gene. (a) Typical *ftz* expression pattern in a wild-type embryo. (b) Embryo microinjected with the XPB antibody. (c) Embryos microinjected with the XPD antibody. Arrows indicate examples of regions lacking the *ftz* mRNA.

complexes occurs in the cytoplasm or in the nucleus. In addition, an interesting and elegant study has shown that transcription activation in the early fly embryo is a gradual and stochastic process mediated by the nucleus to cytoplasm ratio (Pritchard and Schubiger, 1998).

In this work we present evidence that in the cytoplasm of the early *Drosophila* embryo a transitory complex of CAK-XPD, which does not interact with XPB, is present in the cytoplasm. On the other hand XPB and XPD do interact in the cytoplasm of early *Drosophila* embryo. Since XPB and XPD do not physically interact in TFIIH, as this requires p52 and p44 to be assembled in the core of TFIIH (Schultz et al., 2000; Jawhari et al., 2002), we propose that the assembled core complex is present in the cytoplasm of syncytial and cellular blastoderm embryos, but that it does not interact with the CAK-XPD complex. Based on these observations, we also propose that the core complex enters the nucleus independently and then assembles with the CAK in active chromatin domains forming the ten-subunit TFIIH to activate transcription at the mid-blastula transition (Fig. 9). At the mid-blastula transition, most of the CAK complex remains cytoplasmic without interacting with XPD, probably having a function in cell-cycle control (Fig. 9). This model is supported by the fact that the antibody inactivation of either XPB or XPD in early embryos blocks the entry of the two proteins to the nucleus and the onset of transcription. Interestingly, XPD or XPB inactivation does not affect embryo development from syncitial blastoderm to gastrulation, suggesting that development at these stages does not require TFIIH.

In mammalian cells, nuclear entry of a fused GFP-XPD protein is independent of fused GFP-XPB protein and XPD nuclear transport is concluded to be different from that of other TFIIH subunits (Santagati et al., 2001). However, our results suggest that in early fly development, XPD must be assembled with other TFIIH subunits to enter the nuclei. There are examples of transitory precursor complexes that are needed for a factor to enter the nucleus (Lehmann et al., 2002). We propose that this is the case for TFIIH at the onset of transcription in the early *Drosophila* embryo (Fig. 9).

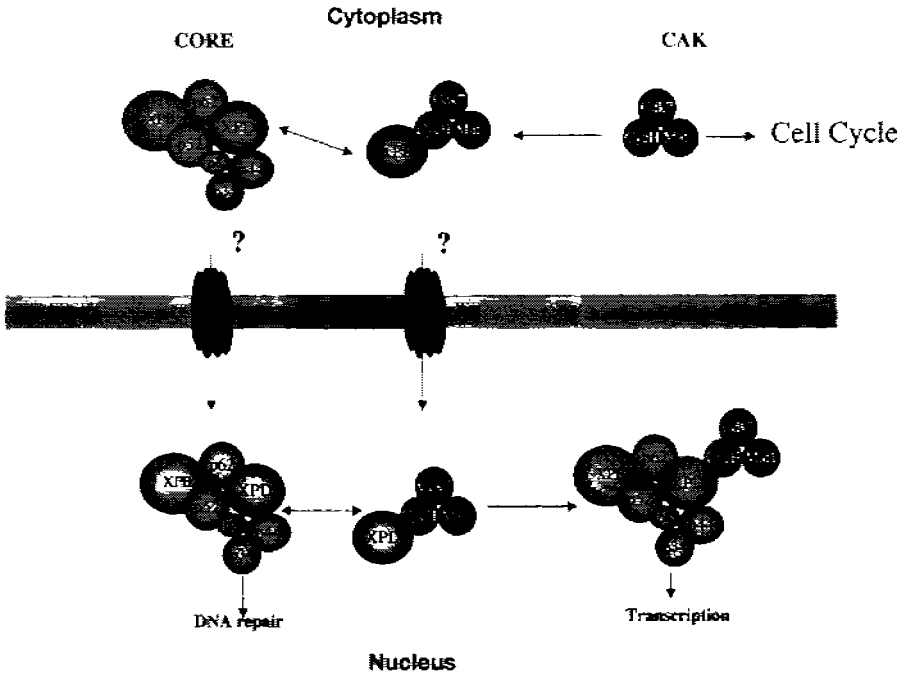

Fig. 9. Nuclear translocation and assembly model of TFIIH at the onset of transcription in the early *Drosophila* embryo.

Maternal RNA encoding some transcription factors that control the establishment of first embryonic coordinates are translated in the syncytial blastoderm and its protein products transported into the nuclei before the mitotic cycle 10 (Hegde and Stephenson, 1993). However, specific gene activation of transcription by these factors only occurs at the onset of transcription after mitotic division ten. The fact the RNA pol II, TBP and other transcription factors migrate into the nuclei before TFIIH suggest that entrance of TFIIH into the nucleus is a key regulatory element to activate transcription at the mid-blastula transition. It is important to remark that these statements agree with the proposed hypothesis of Almouzni and Wolffe (Almouzni and Wolffe, 1995), in which the temporal regulation of transcription during early embryogenesis in some organisms results in a deficiency in the activity of basal transcription activators to the MBT. In addition, our results are supported by the well-known fact that general transcription is inhibited during mitosis and also that in this process many transcription factors are excluded from chromatin (Akoulitchev and Reinberg, 1998; Long et al., 1998).

In conclusion, the CAK and core subcomplexes of TFIIH have differential dynamics in the early fly embryo. At the onset of transcription, the core components are nuclear and most of the CAK remains cytoplasmic. However, a small portion of CAK is positioned at actively transcribed chromatin, in particular at promoters that are activated at the onset of transcription in the early embryo. Later in development, both subcomplexes are preferentially nuclear and co-localize in many chromosomal regions, although we observed a small subset of regions where only the CAK is present. Both the transitory complex CAK-XPD and the core are present in the cytoplasm of the syncytial blastoderm, from where they migrate into the nucleus to form the TFIIH ten-subunit complex and participate together with other components of the basal transcription machinery to initiate transcription.

This study opens several interesting questions for future research. For example, what are the mechanisms that modulate the core and CAK transport between the cytoplasm and the nucleus at the onset of transcription? Why is the CAK preferentially located in the cytoplasm in proliferating cells in the embryo and nuclear in differentiated cells in larval stages? Can the CAK be involved in transcription without interacting with the rest of TFIIH? The eventual elucidation of these questions will be relevant for the understanding of the different functions in which TFIIH is involved.

Materials and Methods

Fly stocks

The w^{1118} *Drosophila* strain was used in all the experiments reported here. Flies were maintained under standard conditions. Embryo collections were performed at 25°C in fly cages using apple-juice agar plates.

Immunofluorescence

Embryos were dechorionated in 50% bleach, fixed in formaldehyde-heptane and devitellinized with methanol. Embryos were permeabilized and blocked in PBST and 4% goat serum for 9 hours. Primary antibodies were added at 1:1000 dilution in TBST and incubated at 4°C overnight. The following primary antibodies were used: Haywire (XPB) (Merino et al., 2002), DmXPD (Reynaud et al., 1999), DmCDK7, human CDK7, human MAT1 and *Drosophila* TBP. After washing in TBST, secondary antibodies (Cy2- or Cy3-conjugated goat anti-rabbit or rat; Rockland) were added at a concentration of 1:1500 in TBST for 1 hour and embryos were washed in TBST. DNA was counterstained with Sytox Green. Co-staining of embryos using an anti-rat-DmXPD polyclonal antibody with anti-rabbit Cdk7 (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-rat XPB (our own preparation) was performed simultaneously. Salivary glands from third-instar larvae were dissected and immunostained as previously described (Reynaud et al., 1999).

Immunolocalization of TFIIH on polytene chromosomes

Fixation and spreading of the chromosomes essentially followed the protocol reported by Engels et al. (Engels et al., 1986) with modifications reported by Reynaud et al. (Reynaud et al., 1999). Co-staining of polytene chromosomes using an anti-rat-Hay or DmXPD polyclonal antibody and an anti-rabbit-CDK7 or MAT1 was performed simultaneously.

Chromatin immunoprecipitation and co-immunoprecipitation from cytoplasmic and nuclear fractions

Embryos at 30-180 minutes of development (~1.0 g) were dechorionated in 3% NaOCl for 2-3 minutes at room temperature. Crosslinking was performed in 1.5 ml of crosslinking solution (PBS, 1.8% formaldehyde) for 15 minutes at room temperature. The crosslinking reaction was stopped by washing thoroughly with PBST (Orlando et al., 1997). Embryos were suspended in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0), with protease inhibitors, and incubated for 10 minutes on ice. Chromatin was sonicated until DNA fragments between of 200 and 1000 base pairs length were obtained. The samples were centrifuged at 14,000 *g* for 10 minutes at 4°C. The supernatant was diluted ten times in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.0, 167 mM NaCl) with protease inhibitors. Samples were taken, the crosslinking reverted and used for the input reaction. Pre-clearing was performed and 1 μg of the antibody was added: anti-DmCDK7 (Santa Cruz Biotechnology); anti-DmXPD (our own preparation); anti-XPB (our own preparation) or anti-RNA pol II-CTD domain (Covance) and the sample incubated for 3 hours at 4°C. As a negative control we used a chromatin extract without antibody. 30 μl of Sepharose-Protein G was then added for 1 hour at 4°C. The antibody-chromatin complex was washed first with high-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 500 mM NaCl), then with LiCl immune wash buffer and twice more with TE. The precipitated complex was suspended in TE and incubated with RNase for 30 minutes at 37°C. The sample was then incubated with 0.5% SDS and 0.5% Proteinase K for 1 hour at 45°C. DNA was extracted with phenol-chloroform several times and precipitated. DNA was used for radioactive PCR using specific oligonucleotides that amplify fragments of ~400 bp for the targets indicated in the results and Fig. 5.

For co-immunoprecipitations (CoIP), cytoplasmic and nuclear fractions were prepared from embryos, about 1-3 g, collected after 1-2.5 hours of development as described in Reynaud et al. (Reynaud et al., 1997). The nuclear preparation was subfractioned to obtain transcriptionally active chromatin (Kamakaka and Kadonaga, 1994). In order to confirm the absence of cross-contamination of cytoplasmic and nuclear fractions, we performed western blots against superoxide dismutase (SOD) enzyme, which is strictly cytoplasmic in both fractions. CoIP was performed according to Leclerc et al. (Leclerc et al., 1996).

XPB and XPD inactivation by injection of specific antibodies and in situ hybridization

To obtain embryos at the correct stage of development (in syncytial blastoderm), flies were allowed to lay eggs on fresh agar trays for 30 minutes. These trays were then removed and embryos were dechorionated. Then embryos were aligned on double-sided sticky tape attached to microscope slides and desiccated for 10 minutes. Embryos were injected dorsally under halocarbon oil. We injected about 1000 embryos with anti-XPD, anti-XPB and anti-Cdk7 antibodies (80 μg/ml). Anti-XPB or -XPD injected antibodies were affinity purified against their respective specific epitopes. Embryos were also microinjected with a control antibody that was affinity-purified mouse anti-GFP polyclonal antibody. After injections, embryos were aged at 25°C for 2.5 hours, washed free of halocarbon oil with 100% heptane, fixed in 100% heptane, 37% formaldehyde (1:1) for 5 minutes. Embryos were then fixed and processed for immunofluorescence as described above. In situ hybridization in microinjected embryos was performed according the standard protocols reported by Mullen and DiNardo (Mullen and DiNardo, 1995).

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DNA Repair and Transcriptional Deficiencies Caused by Mutations in the *Drosophila* p52 Subunit of TFIIH Generate Developmental Defects and Chromosome Fragility[▽]

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The transcription and DNA repair factor TFIIH is composed of 10 subunits. Mutations in the XPB, XPD, and p8 subunits are genetically linked to human diseases, including cancer. However, no reports of mutations in other TFIIH subunits have been reported in higher eukaryotes. Here, we analyze at genetic, molecular, and biochemical levels the *Drosophila melanogaster* p52 (DMP52) subunit of TFIIH. We found that DMP52 is encoded by the gene *marionette* in *Drosophila* and that a defective DMP52 produces UV light-sensitive flies and specific phenotypes during development: organisms are smaller than their wild-type siblings and present tumors and chromosomal instability. The human homologue of DMP52 partially rescues some of these phenotypes. Some of the defects observed in the fly caused by mutations in DMP52 generate trichothiodystrophy and cancer-like phenotypes. Biochemical analysis of DMP52 point mutations introduced in human p52 at positions homologous to those of defects in DMP52 destabilize the interaction between p52 and XPB, another TFIIH subunit, thus compromising the assembly of the complex. This study significantly extends the role of p52 in regulating XPB ATPase activity and, consequently, both its transcriptional and nucleotide excision repair functions.

Several multiprotein complexes are involved in transcription regulation in eukaryotic cells. TFIIH is one of the basal factors that participate in RNA polymerase II (RNA Pol II) transcription (12, 53). Besides this role, TFIIH also makes a fundamental contribution to the mechanism of nucleotide excision repair (NER) and to the control of the cell cycle (6, 12, 14). TFIIH is composed of 10 subunits that can be divided into subcomplexes. The core subcomplex contains six proteins, p8, p34, p44, p52, p62, and XPB, whereas the *C*dk-*a*ctivating *k*inase (CAK) complex includes Cdk7, cyclin H, and MAT1. XPD interacts with members of both complexes and seems to act as a bridge between the core and CAK (12). The CAK subcomplex in *Saccharomyces pombe* and in all higher eukaryotes is involved in cell cycle regulation, acting alone outside the complex (14). TFIIH has several enzymatic activities: XPB and XPD are DNA helicases, Cdk7 is a kinase, and it has been reported that the p44 subunit exhibits a ubiquitin ligase activity (49).

Mutations in either the XPB or XPD gene can lead to three hereditary human disorders: xeroderma pigmentosum (XP), combined symptoms of xeroderma pigmentosum and Cockayne syndrome (XP/CS), and trichothiodystrophy (TTD) (9, 30, 31). Mutations in the p8 protein subunit of TFIIH are also associated with human TTD (19, 39). p8 is important for the stability of TFIIH and has a critical role in DNA repair, where it triggers DNA opening (10).

The TFIIH core subcomplex has a central role in NER: it is required for both global genome repair and transcription-coupled repair (32, 33). In NER, several factors recognize DNA damage caused by UV irradiation. In nontranscribed regions, the DDB and XPC–HR23–β-centrin complexes first bind damaged DNA (48). Then, TFIIH and XPA are recruited; following ATP induction, the helicase activity of TFIIH unwinds the damaged DNA that is ready to be targeted by RPA and XPG/ERCC1-XPF. Following the 5′ and 3′ incisions by XPG and ERCC1-XPF, respectively, the damaged oligonucleotide is removed. The resulting gap is filled by PCNA, RFC, DNA polymerase δ or ε, and a DNA ligase (33, 41). In transcribed regions, NER is achieved by the transcription-coupled repair mechanism. In this case, DNA damage is recognized by a stalled RNA Pol II, which with the help of CSB eventually recruits TFIIH and XPG. Then, a pathway similar to global genome repair is followed (15, 26, 27). A third contribution of TFIIH to genome integrity is its role in the cell cycle. The CAK complex phosphorylates Cdks that are fundamental for cell cycle progression and control (29, 37).

During transcription, TFIIH allows the formation of the open complex through its helicase activities. Cdk7 phosphorylates serine 5 in the heptapeptide repeat of the carboxy-terminal domain of the largest RNA Pol II subunit. It has been proposed that this phosphorylation allows RNA Pol II to escape from the promoter and to start transcribing. However, it

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has recently been documented that in *S. pombe* a mutation in the Cdk7 homologue gene affects only 5% of all fission yeast transcripts, suggesting that the CTD phosphorylation may be compensated for by other kinases (14, 42).

Little is known about the functions of TFIIH subunits other than XPB, XPD, and the CAK subcomplex. For instance, the p44 subunit is important for the assembly of XPD within the core of TFIIH, and it regulates XPD helicase activity (8). Intriguingly, there are no reports of human diseases related to mutations in TFIIH subunits other than XPB, XPD, and p8. It is possible that mutations in these subunits are extremely deleterious. The lack of knowledge reflects a lack of genetic analysis to study the core subunits p62, p52, p44, and p34 in multicellular organisms. In this work, we present a genetic, molecular, and biochemical analysis of the *Drosophila melanogaster* homologue (DMP52) of the human p52 (HP52) subunit. We demonstrate that DMP52 is encoded by the gene *marionette* (*mrn*) and that *mrn* is an essential gene. Defective DMP52 produces specific phenotypes in a complex organism during development and chromosomal instability. This study shows that p52 mutations affect the XPB assembly into TFIIH and reduce XPB ATPase activity. Interestingly, some of the defects observed in the fly caused by mutations in DMP52 resemble some of the clinical features found in patients with alterations in TFIIH.

MATERIALS AND METHODS

***Drosophila* strains.** The *Drosophila* strains used as controls were *OreR*, *W1118*, and *red e* in all the experiments. The P element insertion lines used were *EP3605* (FlyBase identifier [ID] FBti0011698) and *EP0572* (FlyBase ID FBti0007856). The *mrn* alles used were mrn^1, mrn^3, and mrn^5 *red e/TM6B* (FlyBase ID FBgn0002840) and were kindly provided by M. Fuller (16).

UV irradiation sensitivity assay. Third-instar *red e/TM6B*, mrn^1, mrn^3, and mrn^5 *red e/TM6B* and *EP3605/TM6B* larvae were irradiated at different UVB light intensities (joules/m^2) using a UV stratalinker 2400 (Stratagene). Then, the larvae were allowed to develop into adults, and the emerged population was counted.

Complementation test. To perform the complementation test, crosses were performed between the different alleles, including mrn^1, mrn^3, mrn^5 *red e/TM6B*, *EP3605*, and *EP0572/TM6B*. Then, the total number of progeny was determined, and the attended class (genotype) was calculated considering the media of the two major classes observed. In these crosses, we were looking for non-*tubby* progeny. To quantify the larval phenotype, we considered the non-*tubby* larvae and directly evaluated the percentage of tumor generation.

Tissue dissections and staining. The dissection of the larvae, the pharates, and the adult sexual gonads was done in phosphate-buffered saline (PBS). The ovaries were mounted directly in 50% Citifluor (Ted Pella, Inc.), while the testes were frozen in liquid N_2, fixed in MeOH (−20°C) and acetone (−20°C), washed extensively with 0.1% PBS-Tween 20, and mounted in 50% Citifluor. Both sexual gonads were DAPI (4′,6′-diamidino-2-phenylindole) stained (1:10,000) and washed with 0.1% PBS-Tween 20. All the photographs were taken with conventional optic and fluorescence microscopes.

Transgenic flies. The complete wild-type (wt) *Dmp52* cDNA sequence was obtained by reverse transcription-PCR and inserted in the pCaSper *hsp83* vector. The complete wt *hp52* cDNA sequence was amplified by PCR and cloned in the pCaSper *hsp83* vector. The whole genes were sequenced to confirm their integrity. Transgenic flies were constructed following a standard microinjection protocol (47). One *Dmp52* and two *hp52* independent lines in the second chromosome and two *Dmp52* and two *hp52* independent lines in the X chromosome were isolated.

Neuroblast chromosome cytology. Heteroallelic *EP3605*/mrn^1, mrn^3, and mrn^5 and heterozygous *EP3605*/+ organisms were used to obtain mitotic chromosomes from larval neuroblasts following the protocol reported previously (46). Briefly, the brains were dissected in 0.7% sodium chloride and incubated with colchicine for 30 min. Then, the preparation was treated with 0.5% sodium citrate, and the tissue was fixed in 2% formaldehyde-40% acetic acid and squashed. The slides were frozen, dried, and mounted with DAPI. The chromosomes were then visualized in a fluorescence microscope.

Production of recombinant hp52 baculoviruses. Mutations in the *hp52* cDNA were introduced using a site-directed mutagenesis method (36): for mrn^5, a change of A 229 to T (K77/stop); for the mrn^3 allele, a change of C 604 to T (Q202/stop); and for mrn^1, a change of G 928 to A (E310K). New synthetic mutants ware generated by changing G 928 to A plus CG 940 and 941 to GA (E310K/R314D), GC 940 to GA (R314D), and A 929 to G (E310G). The mutant cDNAs were cloned using the appropriate restriction enzymes in pFastBacI vector (Invitrogen), and the whole genes were sequenced to corroborate the mutations. The corresponding recombinant baculoviruses were produced by the Bacto Bac system (Invirrogen). The recombinant viruses were plaque purified, and viral stocks were prepared by three-step growth amplification.

Purification of recombinant TFIIH complexes. Typically, 10^8 cells were infected with combinations of recombinant baculoviruses expressing XPB, XPD, p62, p52, or the different mutant versions, p44, p34, p8, cdk7, cyclin H, and MAT1, as indicated and were collected 48 h after infection. The cells were washed in 1 × phosphate-buffered saline-30% glycerol and subjected to Dounce homogenization in 10 ml of buffer A (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 20% glycerol, 0.1% Nonidet P-40, 5 mM β-mercaptoethanol). After centrifugation at 14,000 × *g* for 30 min at 4°C, the clarified lysates were loaded on a heparin-Ultrogel column (Sepracor) preequilibrated in buffer A. After extensive washing with buffer A containing 300 mM NaCl, the proteins were eluted with buffer A containing 500 mM NaCl. The eluted fractions were dialyzed for 2 h against 50 mM Tris-HCl, pH 7.9, 50 mM KCl, 20% glycerol, 0.1 mM EDTA, and 0.5 mM dithiothreitol and immunopurified using the 1H5 anti-p44 antibody (23).

Transcription and dual-incision NER assays. Runoff transcription (18, 34) and dual-incision assays (1) were carried out as described previously. Briefly, circular DNA containing a single 1,3-intrastrand d(GpTpG) cisplatin-DNA cross-link (Pt-GTG) was prepared as described previously (45). Repair reactions were carried out in buffer containing 45 mM HEPES, pH 7.8, 70 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.3 mM EDTA, 10% glycerol, 2.5 μg of bovine serum albumin, and 2 mM ATP. Each reaction mixture contained 50 ng XPG, 20 ng XPF/ERCC1, 10 ng XPC-hHR23B, 50 ng RPA, 25 ng XPA, and either 1.5 μl of HeLa TFIIH (heparin fraction IV [18]) or the recombinant TFIIH complexes including the p52 wt or mutant subunit. Following preincubation for 10 min at 30°C, 50 ng of Pt-GTG damaged template was added, and the reactions were continued for 90 min at 30°C. The reactions were stopped by rapid freezing. The excised fragments were separated on a 14% urea-polyacrylamide gel after they were annealed with 9 ng of the complementary oligonucleotide and the addition of four α-^{32}P-radiolabeled deoxynucleoside triphosphate (3,000 mCi/mmol) residues by Sequenase V2.1 (USB). Finally, they were visualized by autoradiography as described previously (45).

Interaction assays. Pairwise protein interactions were characterized by coinfection in Sf9 cells (2.5×10^7) with the corresponding recombinant baculoviruses at a multiplicity of infection of 5. The cells were collected 48 h after infection, washed in 1× phosphate-buffered saline–30% glycerol, and subjected to Dounce homogenization in 2.5 ml of buffer A. The clarified lysates were obtained by centrifugation at 14,000 × *g* for 30 min at 4°C. Fifty microliters of clarified lysate was adsorbed on 20 μl of protein G-Sepharose beads linked with the appropriate monoclonal antibody (1B3, which recognizes the ATP binding site of XPB, or 1D11, which recognizes the N terminus of p52) in buffer containing 20 mM Tris-HCl, pH 7.5, 50 mM KCl, 10% glycerol, and 0.1 mM EDTA. After 1 h of incubation at 4°C, the beads were washed extensively in buffer C containing 150 mM KCl and resuspended in Laemmli buffer. The proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12.5% acrylamide) and revealed by Western blotting using the appropriate monoclonal antibodies (23).

ATPase assay. Protein fractions were incubated for 2 h at 30°C in the presence of 1 μCi [γ-^{32}P]ATP (7,000 Ci/mmol; ICN Pharmaceuticals) in a 20-μl reaction volume in 20 mM Tris-HCl, pH 7.9, 4 mM $MgCl_2$, 1 mM dithiothreitol, 50 mg/ml bovine serum albumin, and, when indicated, 120 ng of supercoiled double-stranded DNA (pSK). The reactions were stopped by adding EDTA to 50 mM and sodium dodecyl sulfate to 1% (wt/wt). The reaction mixtures were then diluted fivefold, spotted onto polyethylenimine thin-layer chromatography plates (Merck), run in 0.5 M LiCl-1 M formic acid, and autoradiographed.

Immunohistochemistry and quantification of fluorescence in nuclear sections of salivary glands. Salivary glands from third-instar larvae, wt and heteroalelic combinations between the *EP3605* and mrn^1, mrn^3, and mrn^5 alleles, were dissected and immunostained as previously described (40). For quantification of fluorescence, we followed the protocol reported previously (40). Briefly, we used confocal microscopy to visualize XPD, XPB, TBP, and histone fluorescence. Representative images of protein fluorescence in nuclear sections from the wt

and each genotype of *Dmp52* were obtained. The nuclear area (156 pixels for each nucleus) was analyzed for each genotype using a photon-counting program. The protein fluorescence frequencies were obtained as a histogram. The relative fluorescences were shown in a bar chart, with the average frequencies of XPB-TBP, XPB-histones, XPD-TBP, and XPD-histones in the *y* axis and each genotype of p52 in the *x* axis. Standard errors were indicated in each plot.

RESULTS

The gene *marionette* encodes the *Drosophila* p52 homologue. In *Drosophila*, the gene *marionette* (*mrn*) was identified in a genetic screen for enhancers of mutants in the β-tubulin 85D gene (β-tub85D) (16). Because *mrn* alleles fail to complement alleles of β-tub85D and do not map to the β-tub85D locus (16), the mutations define a new locus. *mrn* maps at position 71C3-E5, which is the same position where the homologue for the *Drosophila* p52 gene (*Dmp52*) has been localized, so we hypothesized that *mrn* and *Dmp52* were the same gene.

To confirm that *mrn* encodes the DMP52 protein, we analyzed two reported EP element insertions that are in or near *Dmp52*. The transposon *EP3605* (FlyBase ID FBti0011698) is inserted 20 nucleotides upstream of the predicted translation initiation codon for *Dmp52* (Fig. 1A). The homozygous flies are viable, although adult organisms are sterile and have several morphological defects (see below), suggesting that the *EP3605* mutant is hypomorphic for *Dmp52*. *EP0572* (FlyBase ID FBti0007856) is inserted inside the first exon of *Dmp52*, 5 nucleotides downstream of the translation initiation codon; it is lethal in the homozygous condition. Heteroallelic flies with both EP insertions are also lethal, suggesting that both insertions affect the same locus (Fig. 1B).

Several ethyl-methane-sulfonate mutant alleles of *mrn* have been identified (FlyBase ID FBgn0002840) (16). We performed complementation experiments among them and with the EP insertion alleles. In particular, we analyzed the mrn^1, mrn^3, and mrn^5 alleles, which are all lethal when homozygous (Fig. 1B). None of the *mrn* alleles were able to complement each other, and all failed to complement the *EP0572* lethality (Fig. 1B). On the other hand, heteroallelic flies carrying any *mrn* allele and the *EP3605* transposon are semilethal, with the penetrance of the lethality depending on the heteroallelic combination. In mrn^5/*EP3605* flies, only 5% of the expected class survives, while in mrn^3/*EP3605* flies, 80% of the expected class of flies develop into adults; in both cases, the adults are sterile (Fig. 1B). In some of the allelelic combinations, a proportion of the individuals develop only up to the third-instar larval (Fig. 1C) or pupal stage. These organisms present melanotic tumors before dying. We hypothesize that the wt *mrn* product of maternal origin supports variable levels of embryo development.

To confirm that *mrn* alleles affect the predicted DMP52 protein, we sequenced the *Dmp52* mrn^1, mrn^3, and mrn^5 alleles. In mrn^5, a change of C 286 to T introduces a stop codon (Q96/stop) (Fig. 1E). In the case of the mrn^3 allele, a change of C 673 to T also introduces a stop codon (Q225/stop). Therefore, these two mutants, mrn^3 and mrn^5, are predicted to result in truncated DMP52 peptides, one of 95 amino acids and the other of 224 (Fig. 1E). In the case of the mrn^1 allele, we found a change of G 1018 to A, which changes a glutamic acid to lysine (E340K). The glutamic acid affected in the mrn^1 allele is highly conserved in all p52 homologues accessible from GenBank, and it is within a region that is important for the interaction of p52 with XPB in human TFIIH (23). Based on molecular analysis of these three *mrn* alleles and the complementation experiments with the EP insertions that affect *Dmp52*, our results prove that *mrn* is *Dmp52*.

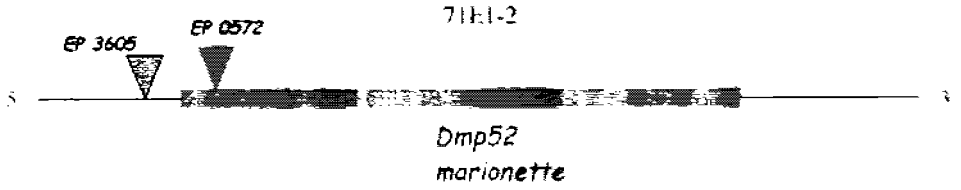
Heterozygous mutants in *DmXPB* (*haywire*) and *DmXPD* are more sensitive than the wt to UVB irradiation (35, 38, 44); thus, we predicted that the *Dmp52* mutants should also be more sensitive than the parental strain. Adult heterozygous *Dmp52* mutants (mrn^1, mrn^3, and mrn^5 alleles), as well as a control strain that has the same genetic background and the same balancer chromosome, were irradiated with UVB for various times at a fixed irradiance (see Materials and Methods). The plot presented in Fig. 1D clearly shows that flies heterozygous for *Dmp52* lethal alleles are more sensitive to UVB irradiation than the control strain; in particular, the mrn^1 mutant is the most sensitive to UVB irradiation among all the tested *Dmp52* alleles. The increased UVB irradiation sensitivity is most striking at 100 J/m^2, the lowest dosage examined. It is important to stress that one wt *Dmp52* allele is present in the balancer chromosome in the analyzed flies, and therefore, all the analyzed mutants were heterozygous.

Developmental phenotypes associated with *Dmp52* mutations in the fly. As we mentioned above, some of the homozygous *Dmp52* mutants and heteroallelic combinations die at the stages of third instar larvae and pupae. Interestingly, we noticed that both larvae and pupae homozygous for mrn^1, mrn^3, and mrn^5 alleles and the heteroallelic combinations with the *EP3605* mutant are smaller than heterozygous or wt organisms (Fig. 2A and B). This phenotype resembles the minute phenotype, usually caused by protein translation defects. Both larvae and pupae have melanotic tumors in diverse places (Fig. 2F and G). These tumors are aggregates of hematopoietic cells that result from the overproliferation of blood cells and are considered a leukemia-like phenotype (52). In addition, we found that in the specific combination of the mrn^3 or mrn^5 allele with *EP3605*, there is a highly reproducible and specific melanization in the larval intersection between the midgut and posterior gut in a region known as the imaginal ring (Fig. 2H and I). The melanization in the imaginal ring could be necrosis, or it could result from abnormal growth of tissue that is subsequently melanized. Organisms that survive the larval stages reach different pupal stages before dying (Fig. 2C). Flies that die at the pharate stage have deformations in the cuticle and exhibit the brittle-bristle phenotype (Fig. 2D and E). These two phenotypes (cuticle deformations and brittle bristles) are identical to those previously reported in *haywire* (*DmXPB*) mutants and strongly suggest that these are TFIIH-related defects (35).

The heteroallelic organisms that carry the EP insertion *EP3065* and point mutation alleles are semilethal. Interestingly, the organisms that are able to develop to adulthood are smaller than a wt fly, resembling a viable minute phenotype (Fig. 2J). Additionally, these flies have both cuticular-deformation and brittle-bristle phenotypes (data not shown). Although some of the *EP3065*/*mrn* (mrn^1, mrn^3, and mrn^5 alleles) heteroallelic flies can develop into adults, they are sterile, as are the homozygous *EP3065*/*EP3065* flies.

Animals were analyzed to determine if sterility was the result of defective gametogenesis. In the case of females, two pre-

A



B

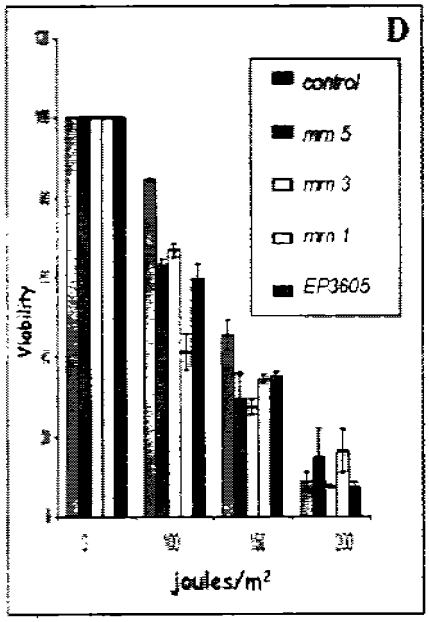
Complementation test between different Dmp52 alleles

	EP0572	mrn1	mrn3	mrn5
EP3605	0% (397)	61% (482)	71% (352)	5.1% (799)
EP0572	0%	0% (258)	0% (586)	0% (489)
mrn1		0% (226)	0% (309)	0% (546)
mrn3			0% (252)	0% (716)
mrn5				0% (572)

C

Frequency of larval tumor phenotypes in Dmp52 heteroallelic combinations

	mrn1	mrn3	mrn5
EP3605	Larvae with general necrosis	14% larvae with melanotic tumors	88% larvae with melanotic tumors
mrn1	Some larvae with melanotic tumors	11.6% larvae with necrosis or melanotic tumors	



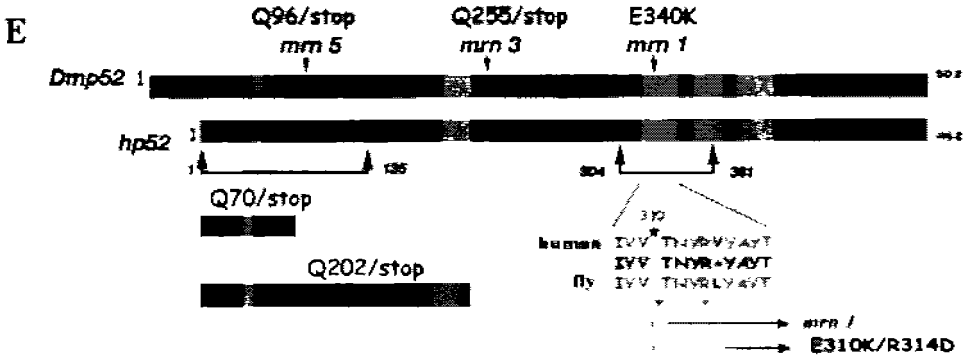


FIG. 1. Genetic and molecular description of *Dmp52* alleles, complementation tests, and UVB irradiation sensitivity assay. (A) Genomic organization of the *Dmp52* (*mrn*) gene and EP insertions. *Dmp52* contains two coding exons and a short intron. *Dmp52* maps in the cytological position 71E1-2. The positions of the two EP insertions are shown by inverted triangles. *EP3605* is inserted at the 5′ end of the gene 20 nucleotides from the start codon. *EP0572* is inserted in the first intron of the *Dmp52* gene, 5 nucleotides after the start codon, disrupting the DMP52 protein. (B) Complementation test between different *mrn* point mutations and the EP insertions. The percentage represents the adult flies observed in the expected class. The number in parentheses is the number of expected individuals for each class. Only *EP3605* is able to complement, at least partially, the three different *mrn* point mutation alleles. The other allele combinations are lethal, including *EP0572*. (C) Frequency of larval tumor phenotypes in the *Dmp52* heteroallelic combinations. The percentage represents the larvae observed to have any tumor compared to the healthy class. In the case of the *EP3605* allelic combinations, the unaffected populations are presumably those that continue development until adulthood. The larvae of the allelic combinations including at least one copy of *Dmp52* (E340K) mrn^1 were not quantified. (D) Viability test of the *Dmp52* mutants and the parental strain after different UVB irradiation doses. Third-instar larvae heterozygous for *Dmp52* mutants were irradiated and then allowed to develop until adulthood. The survival rate is indicated for each strain. The graph represents the results of at least six independent dosage experiments for each genotype. The statistical analysis by analysis of variance indicated a P value of <0.0001 for each mutant compared with the parental strain at 100 and 150 J/m^2. The error bars indicate standard errors. (E) Molecular characterization of the *Dmp52* point mutants and diagram showing the mutations characterized in the *Dmp52* gene introduced into *hp52*. The gray boxes indicate the regions with highest similarity between the DMP52 and HP52 proteins. The amino acid changes found in the mrn^1 (E340K), mrn^3 (Q225/stop), and mrn^5 (Q96/stop) alleles are indicated by arrows. Truncated peptides, as well as the single and double amino acid changes analyzed in the HP52 protein, are also indicated.

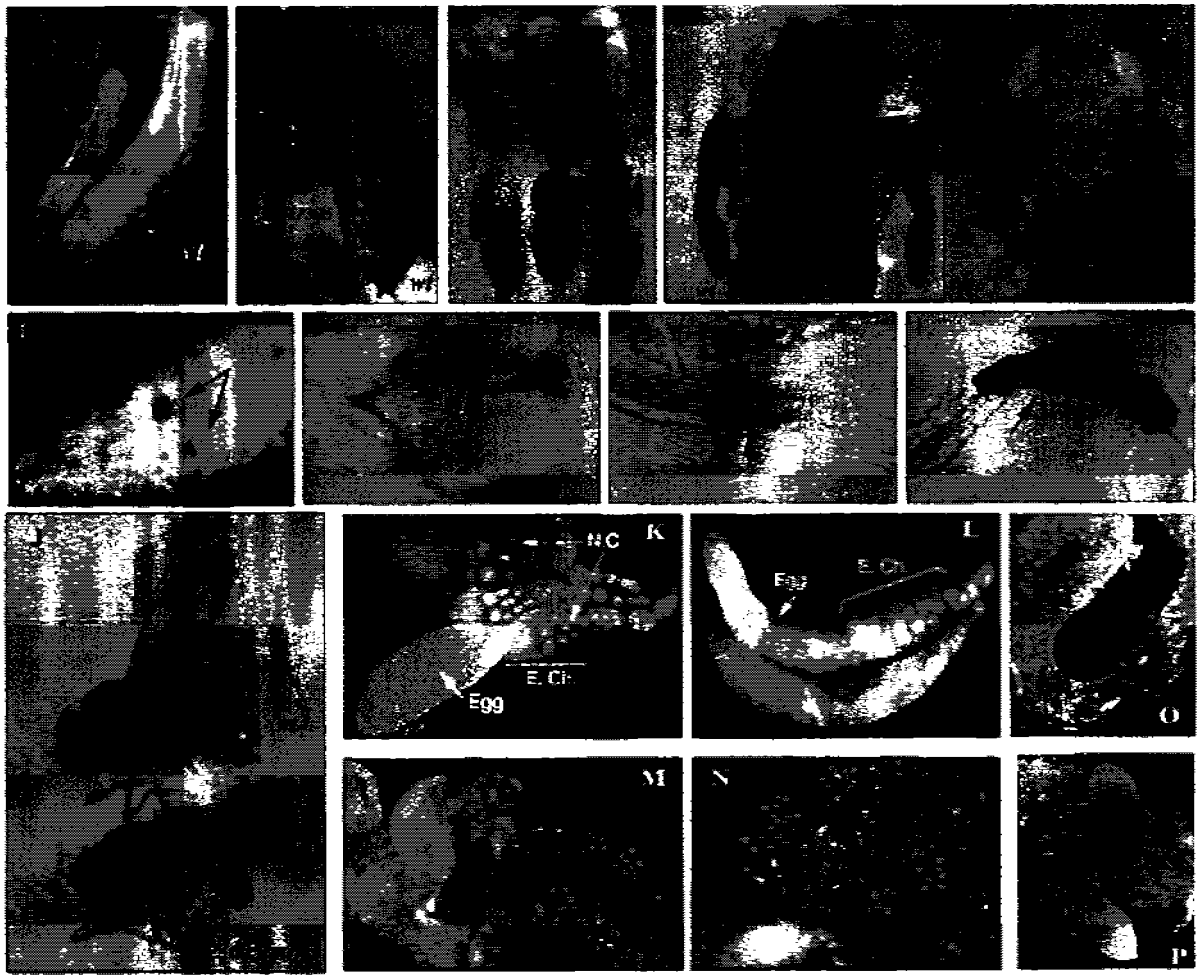


FIG. 2. Developmental phenotypes associated with mutations in *Dmp52*. (A) Wt and mrn^1/mrn^1 mutant third-instar larvae. Note the reduced side of the mutant organism compared to the wt. (B) Wt and *EP3605/EP3605* mutant pupae. Note the reduced size of the mutant pupa. (C) Some *EP3605/EP3605* and EP3605/mrn^1, mrn^3, and mrn^5 flies can go through metamorphosis but die at different stages before completing development. (D and E) Wt and *EP3605/EP3605* pharates, respectively. The mutant organisms have cuticular defects, as well as fragile and deformed bristles (brittle-bristle phenotype). The defects are indicted by arrows. (F and G) mrn^1/mrn^1 and heteroallelic *EP3605*/mrn^3 larva and pupa, respectively, with melanotic tumors indicated by arrows. (H and I) Presence of melanization in the imaginal ring midgut of heteroallelic *EP3605*/mrn^3 larvae (arrows). This phenotype was always present in the imaginal ring region of the larval gut and extended from this point in both directions. (J) Wt and *EP3605*/mrn^3 adult females. Each genotype is indicated. Note the difference in size between the organisms. (K) Ovarioles from a heterozygous *EP3605*/+ female. Note that the egg chambers (E. Ch.), nurse cells (N.C.), and oogenesis stages are normal. (L) Ovarioles from an *EP3605/EP3605* female. Note that the first oogenesis stages are normal, but the eggs are deformed, apparently resulting from an aberrant chorion (egg shell). (M and N) Ovarioles from heteroallelic *EP3605*/mrn^1 and *EP3605*/mrn^3 females, respectively. Note the aberrant egg chambers, the disrupted nurse cells, and the presence of picnotic nuclei in the eggs (indicated by arrows). (O) Testis from a heterozygous mrn^1/+ male. Note that all spermatogenesis stages are present. (P) Testes from an *EP3605*/mrn^1 organism. Note that the testes are aberrant and lack cells progressing through spermatogenesis. The tissue preparation nuclei were visualized by DAPI staining.

dominant gametogenesis defects were observed. Figure 2K shows egg chambers with normal oogenesis development in a heterozygous +/mrn^3 female. In contrast, in ovarioles from an *EP3605/EP3605* female, the first oogenesis stages are normal, but the mature oocytes are deformed (indicated as eggs in Fig. 2L), apparently resulting from an aberrant chorion (egg shell), and therefore the eggs are not viable. In ovarioles from heteroallelic *EP3605*/mrn^1 and *EP3605*/mrn^3 females, aberrant egg chambers were observed and picnotic nuclei containing what looked like apoptotic bodies were present in the nurse cells (indicated in panels M and N), and therefore oogenesis was not completed. In heteroallelic *EP3605*/mrn^1 males, defective testes were present, and spermatogenesis was absent or not completed in most of the organisms (Fig. 2O and P).

Dmp52 is thus similar to *haywire (hay)*, which has a defined deficiency in TFIIH and specific developmental defects. Because *Dmp52* fly mutants exhibit specific developmental phenotypes, we infer that some developmental processes are more sensitive than others when TFIIH is subfunctional (21, 35, 53).

The HP52 homologue partially rescues some phenotypes produced by mutations in DMP52. Although it has been reported that the human XPB homologue is not able to rescue mutations in *haywire Drosophila* mutants, even if its similarity is 71% (DmXPB) (24), we nevertheless explored whether HP52,

TABLE 1. Rescue of the lethality of homozygous *mrn* alleles by different transgenic flies

Mutant genotype	Lethality rescue by transgenic fly (%)[a]:				
	Dmp52-1	*Dmp52-1/ Dmp52-2*	*Dmp52-mut*[b]	*hp52-2*	*hp52-1/ hp52-3*
mrn^1/*mrn*^{1}	2.4 (540)	3.4 (256)	0 (200)	0 (262)	2 (111)
mrn^3/*mrn*^{3}	5.0 (592)	6.0 (168)	0 (190)	1.5 (126)	3.5 (109)
mrn^5/*mrn*^{5}	10.4 (281)	15.0 (267)	NA	NA	7.8 (153)

[a] The percentage represents recovery of homozygous flies compared to the expectation for full complementation (number in parentheses). NA, not analyzed.

[b] The *Dmp52-mut* line is a transgenic line expressing the double point mutant (E340K-R34YE).

TABLE 2. Rescue of the sterility phenotype in heteroallelic organisms by different transgenic flies

Mutant genotype[a]	Sterility rescue by transgenic fly (%)[b]				
	Dmp52-1	*Dmp52-2*	*hp52-3*	*hp52-4*	*Dmp52-mut*[c]
EP3605/EP3605	85	76	95	59	0
mrn^5/*EP3605*	75	86	100	77	0
mrn^1/*EP3605*	100	80	0	0	0
mrn^3/*EP3605*	75	NA	75	87	NA

[a] Homozygous *EP3605/EP3605* and heteroallelic (*EP3605/mrn*^{1}, *mrn*^{3}, and *mrn*^{5}) adults are 100% sterile.

[b] The percentage represents the individual flies that were fertile and able to generate progeny that developed to larval stage. The total numbers of flies tested were between 10 and 100 for each condition. The numbers of males and females are merged to give a general percentage. There was not bias in sex ratios (data not shown). NA, not analyzed.

[c] The *Dmp52-mut* line is a transgenic line expressing the double point mutant (E340K-R344E).

which has 50% identity to DMP52, could rescue the mutants that affect *Dmp52*. To conduct this test, we constructed transgenic flies that express either *Dmp52* or the *hp52* full-length cDNA under the control of the *DmHSP83* promoter using the Casper vector (see Materials and Methods). This promoter is constitutive, and we expected to have moderate expression of the transgenes in all cell types throughout development. We performed the rescue experiments by making crosses between heterozygous point mutation and *EP3605* strains with different transgenic lines and then deriving flies homozygous for the *Dmp52* mutation and heterozygous for the transgene. First, we looked for rescue of the lethality phenotype. Table 1 shows that, although some homozygous point mutants can develop to adulthood with one copy of the wt *Dmp52* gene, the percentage of rescue is low, but as expected, it increases with two transgene copies. The lack of a complete rescue by a single copy of the *Dmp52* transgene can be explained if *Dmp52* expression is not optimal; components necessary for normal transcript levels could be missing from the promoter or the cDNA or reflect the lack of natural genomic context. In addition, it is known that low levels of transgene transcripts can be influenced by the surrounding chromatin.

In contrast, the sterility phenotype of homozygous *EP3605* flies is completely rescued by a single copy of the *Dmp52* transgene (Table 2). As mentioned above, the *EP3605* mutant is hypomorphic; we expect that there are reduced *Dmp52* transcription levels because of the position of the EP. Therefore, although transgene expression is probably not optimal, it is sufficient for the complete rescue of milder phenotypes.

In the test of complementation using the *hp52* cDNA, a partial rescue of lethality was also observed in homozygous point mutations, but as expected, it was lower than with the *Dmp52* transgene (Table 1). Note that two independent transgenic *hp52* fly lines rescued, at least partially, the *mrn* lethal phenotype (Table 1). These lines have a single transgene, and also, the value increases with two copies. Interestingly, with the exception of the *mrn*^{1}/*EP3605* flies, the *hp52* transgene was able to fully rescue the fertility defect in homozygous *EP3605* flies (Table 2). Note that a *Dmp52* double point mutant (E340K/R344D) (Fig. 1E; also see below) transgenic fly was unable to rescue the phenotype in any case. These results demonstrate that the human homologue of *Dmp52* is sufficiently conserved to rescue at least some *Dmp52* functions during development. The inability of *hp52* transgenes to substitute for the *mrn*^{1} allele that contains only a single amino acid change is intriguing. Therefore, a more detailed analysis of the protein-protein interactions between p52 and other TFIIH subunits will be required to address this point.

***Dmp52* mutations affect nuclear TFIIH levels in vivo.** Considering the phenotypes of the fly, we wondered whether these defects could be associated with some particular TTD features. Knowing that reduction in the TFIIH cellular concentration is a typical TTD phenotype (4), we analyzed third-instar salivary gland cells from heteroallelic flies for *EP3605*, either the *mrn*^{1}, *mrn*^{3}, or *mrn*^{5} allele, by immunofluorescence of XPB and XPD. Internal controls were made using anti-histone H3 and anti-TBP antibodies. We found that in all the *Dmp52* heteroallelic combinations, there was a substantial reduction of the DmXPB protein levels (Fig. 3A and B). With the exception of *EP3605/mrn*^{5} organisms, we found that XPD levels were also reduced when *Dmp52* was mutated (Fig. 3C and D). This finding parallels the observations reported in XP, XP/CS, and TTD patients, where some of the XPB, XPD, and p8 mutations affect the cellular levels of TFIIH.

Chromosomal instability in *Dmp52* mutants. TFIIH is implicated in NER, which is fundamental for genome stability. In humans, mutations in the XPD subunit are linked to cancer. As we have demonstrated in this work, *Dmp52* mutants are deficient in DNA repair and as a consequence may have chromosome fragility. Therefore, it was interesting to analyze whether mutations in *Dmp52* could generate chromosomal instability. To examine chromosomal integrity, we used neuroblast squashes from heteroalleic *Dmp52* mutants and control larvae. In mitotic spreads from *Dmp52* mutants (Fig. 4D to I), we observed a high rate of chromosomal aberrations that were evident when comparing wt metaphasic chromosomes (Fig. 4A to C). The most common aberrations were fragmentation of chromosomes and partial chromosome condensation. Acentric chromosome fragments were observed, as well as loss of chromosomal arms (indicated in Fig. 4F, G, and H). In addition, chromosome association and chromosomal rearrangements were found (Fig. 4F and G). These results show for the first time in *Drosophila* that mutations in one of the TFIIH subunits affect chromosomal integrity.

Effect of *Dmp52* point mutations on the XPB ATPase activity and the TFIIH transcription and DNA repair activities. We next asked how the *Dmp52* mutations could affect TFIIH functions. The *Dmp52* point mutations were introduced by site-

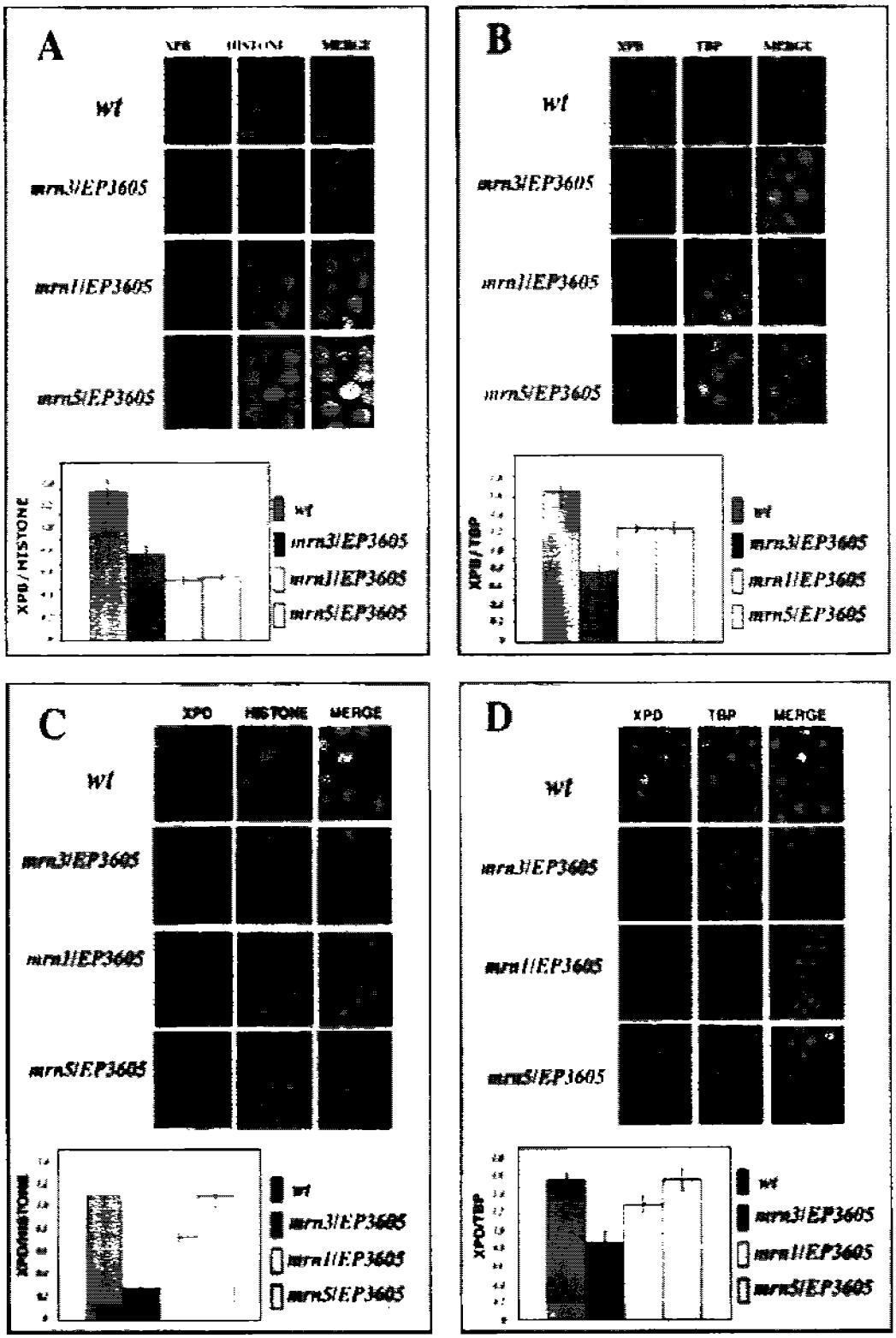


FIG. 3. XPB and XPD protein levels are reduced in the *Dmp52* mutants. Wt and heteroallelic mutant third-instar larval salivary glands were costained with either XPB/histone H3, XPB/TBP, or XPD/histone H3 and XPD/PBT antibodies. (A) Costaining and quantification of the XPB/histone ratio. Note that the XPB levels are reduced at least twofold in the mutant nuclei. (B) Costaining and quantification of the XPB/TBP ratio. XPB levels are reduced compared with the control. (C) Costaining and quantification of the XPD/histone ratio. (D) Costaining and quantification of the XPD/TBP ratio. In *EP3605/mrn*^{1} and *EP3605/mrn*^{3} heteroallelic flies, the XPD levels are reduced. For quantification of fluorescence, we followed the protocol reported previously (31) (see Materials and Methods). At least 10 nuclei for each condition were analyzed. The error bars indicate standard errors. For details, see the text.

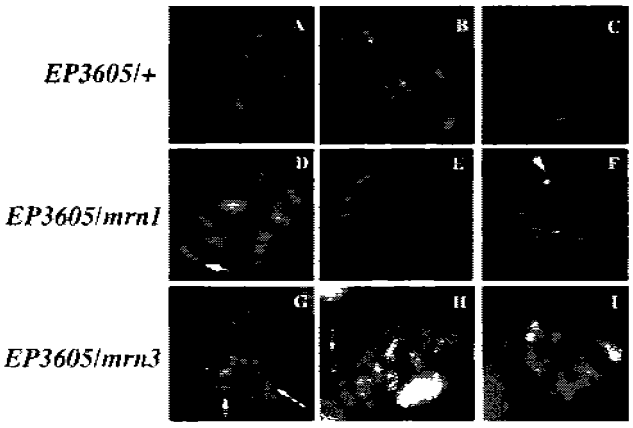


FIG. 4. Mutations in *Dmp52* cause chromosomal aberrations. (A to C) Mitotic spreads from *EP3605/+* neuroblasts, which are identical to the wt. (D to F) Mitotic spreads from *EP3605/mrn*^{1} heteroallelic mutants. Note the presence of partially condensed chromosomes and chromosomal fragments, indicated by the arrows. (G to I) Mitotic spreads of *EP3605/mrn5* heteroallelic mutants. Note the presence of chromosomal fragments, indicated by arrows, and partially condensed chromatids.

directed mutagenesis into the *hp52* cDNA at positions Q70/stop (corresponding to *mrn*^{5}), Q202/stop (corresponding to *mrn*^{3}), and E310K (corresponding to *mrn*^{1}), as well as at positions R314D and E310K/R314D, a single and a double mutation (see Discussion) located in a highly conserved region, in order to generate baculoviruses expressing the corresponding proteins. The truncated p52 peptide derived from the Q70/stop mutant was unstable in Sf2 cells (data not shown) and was not analyzed further. Knowing that p52 interacts with XPB (23) and stimulates its ATPase activity to trigger NER (F. Coin, V. Oksenych, and J. M. Egly, submitted for publication), we were wondering what would be the consequences of p52 mutations for XPB ATPase activity. Immunoprecipitations were carried out using insect cell extracts expressing the mutated HP52 proteins together with the wt XPB. p52-E310K and p52-E310K/R314D mutations significantly weakened XPB binding compared to wt p52 or p52-R314D (Fig. 5A). We also observed that the truncated p52-Q202/stop was hardly retained on XPB. There was also a similar reduction of the p52/XPB ratio when we used the p52 antibody to pull down p52 (data not shown). As a consequence, when tested in an ATPase assay, we found that XPB was weakly stimulated by p52-E310K (22%) and p52-E310K/R314D (17%), while wt p52 and p52-R314D were stimulated normally (Fig. 5B). Taken together, our results indicate that the mutations found within a highly conserved region in *Drosophila* p52 prevent the regulation of XPB ATPase by the p52 regulatory subunit.

To further analyze the consequences of p52 mutations in transcription and DNA repair, recombinant IIH10 (rIIH10) complexes containing either the wt or modified p52 protein, together with the other nine TFIIH subunits, were produced in H5 insect cells and subjected to a two-step purification process (11). The rIIH10 complexes immunoprecipitated with antibodies directed toward the p44 subunit of TFIIH were next analyzed by Western blotting after being washed with 300 mM KCl (Fig. 5C). The E310K, E310K/R314D, and R314D-p52 proteins were incorporated into the IIH core, as demonstrated by the presence of p62 and cdk7 subunits. However, XPB incorporation was significantly reduced in rIIH10/p52-E310K/R314D compared with rIIH10/p52-wt. It is intriguing that XPB does not play a major role in the stability of rIIH10.

These mutant rIIH10 complexes were tested in both reconstituted transcription and NER assays (11). We observed that the transcription and NER activities of rIIH10/p52-E310K/R314E were significantly reduced compared to rIIH10/p52-wt (Fig. 5D, lanes 7 to 9), underlining how the preservation of the HP52/XPB interacting domains is crucial for the maintenance of both activities. These results also explain why *Dmp52* transgenic flies containing this double mutation could not rescue the wt phenotype in *Drosophila* (Tables 1 and 2). We also noticed that the transcription activity of rIIH10/p52-E310K (which produced the severe phenotype in the fly) was partially (around 25%) inhibited, although NER was rather normal compared to rIIH10/p52-wt (lanes 4 and 5). rIIH10/p52-E314K did not exhibit any deficiency in either transcription or NER activities (lanes 10 to 12).

DISCUSSION

Disturbing TFIIH activities and architecture. It has previously been demonstrated that HP52 is necessary for the incorporation of XPB into the core of TFIIH (23). Based on this information and because HP52 can rescue some of the DMP52 mutant phenotypes, we introduced mutations into *hp52* cDNA to reconstruct the *Drosophila* mutations and then analyzed their effects in vitro. Here, we have shown that mutations in a highly conserved domain of DMP52 prevent the binding and regulation of XPB ATPase activity, a function that is required for both transcription and DNA repair. With such findings, is intriguing that the p52-E310K mutation that confers UV sensitivity on the fly does not result in an appreciable in vitro biochemical phenotype in humans, although we demonstrated that the mutation does disturb the regulation by p52 of XPB ATPase activity. It thus seems that in humans, the other subunits of TFIIH may circumvent the weakening of the XPB/p52 interaction, allowing TFIIH to be active in both transcription and DNA repair, at least in our in vitro assays. In *Drosophila*, the other TFIIH subunits are probably unable to compensate for the XPB-p52 interaction defect. However, the importance of residue E310 of p52 for TFIIH transcription and repair functions became clear when a mutation in this residue (E310K) was combined with a mutation in another very conserved residue of p52 (R314E) located 4 amino acids downstream. In this case, p52 was not able to anchor XPB to the core TFIIH, resulting in a TFIIH with low repair and transcription activities. These results show that the region where the HP52/E310R-R314E mutant is located is very important for the interaction with XPB and its subsequent assembly or retention in TFIIH (Fig. 5). The double mutant E310K/R314E does not exist in the fly or human; its construction was based on the high conservation of the residues E310 and R314 in all organisms. Of course, the key element was the mutant *mrn*^{1}, which has the substitution E310K. As mentioned above, this region binds with XPB, and our results indicate that changes in the amino acid sequence in this domain have a more deleterious effect than a deletion that does not contain this region.

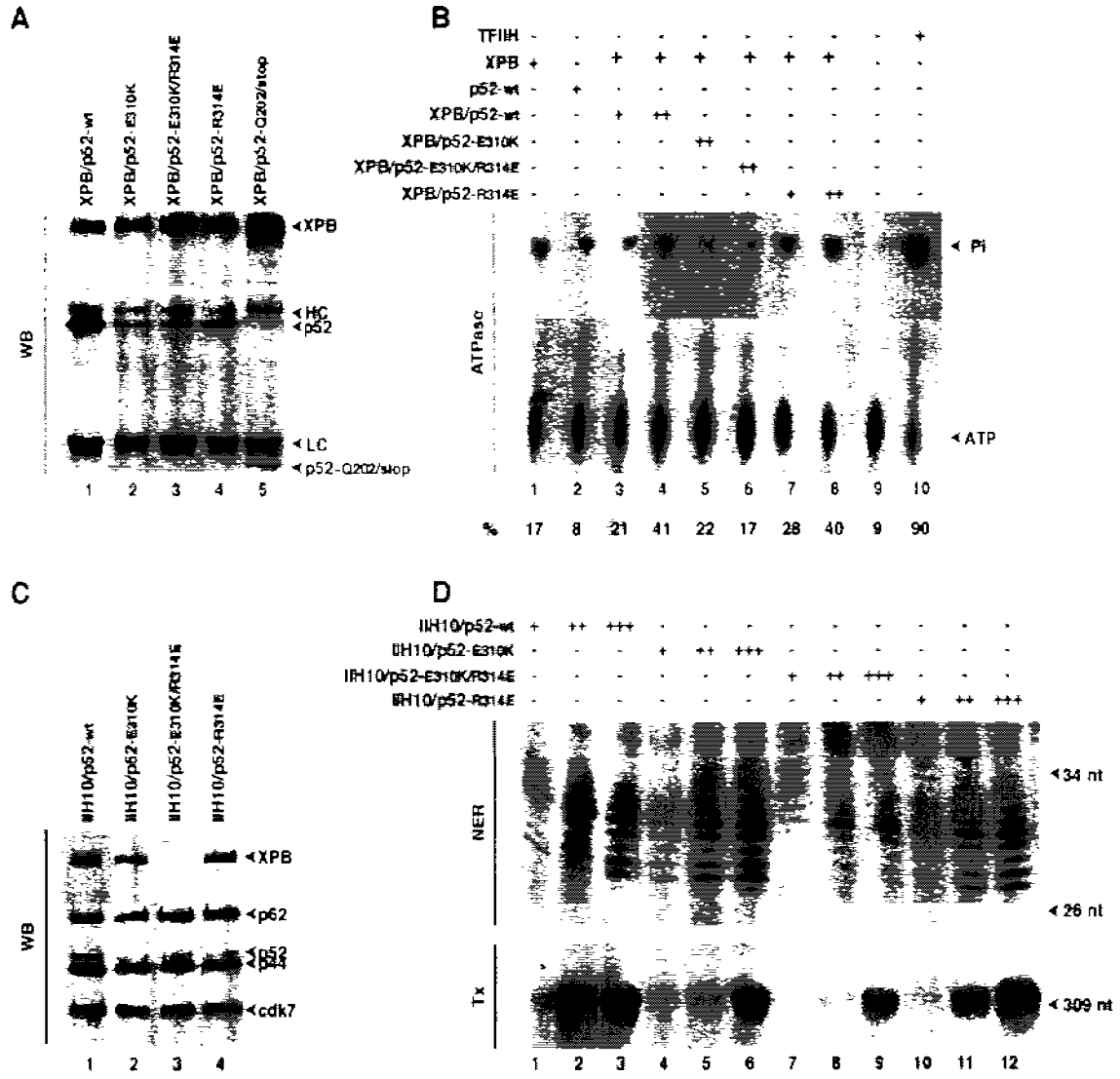


FIG. 5. Mutations in HP52 cause low stimulation of XPB ATPase activity and NER defects. (A) Recombinant XPB and mutant HP52 expressed in insect cells were immunoprecipitated with an anti-XPB antibody and analyzed by Western blotting (WB). The positions of XPB, HP52, the truncated p52-Q202/stop, and the heavy (HC) and light (LC) immunoglobulin chains are indicated. (B) Wt XPB, either alone or in combination with the p52 mutants, was immunoprecipitated with Ab-XPB antibody immobilized on agarose beads. The immobilized protein complexes were washed with 0.4 M KCl and tested in an ATPase assay. TFIIH was used as a control. Quantifications of the inorganic phosphate (P_i) release and ATP were done with a Bio-imaging analyzer, and the ratio presented at the bottom of the figure shows the percentage of the released P_i. (C) Immunopurified recombinant TFIIHs (as indicated at the top of the blot) from insect cell extracts containing all the TFIIH subunits, including either wt HP52 or mutant HP52, were subjected to Western blot analysis using antibodies against XPB, p62, p52, or Cdk7. The TFIIH subunits are indicated by arrowheads. (D) Fifty, 100, and 200 ng of the various IIH10 complexes were tested in a dual-incision assay (NER) containing the recombinant XPC-HR23b, XPA, RPA, XPG, and ERCC1-XPF factors and a closed-circular plasmid containing a single Pt-GTG–DNA cross-link as a template or in a reconstituted transcription assay (Tx) composed of recombinant TFIIB, TFIIF, TBP, TFIIE factors, the purified RNA Pol II, and the adenovirus major late promoter template (11). The sizes of the incision products or transcripts are indicated. nt, nucleotides.

TTD-like phenotypes in *Dmp52* mutants. In the case of the p52 subunit, there are only reports of a conditional mutant in yeast (13), a model in which no developmental effects can be studied. We analyzed two different types of mutations in the *Drosophila* p52 homologue, EP insertions and three point mutations. With the exception of the *EP3605* insertion, all of the analyzed mutants are homozygous lethal. Heteroallelic combinations of *EP3605* with the point mutant alleles are semilethal, and adult organisms showing intriguing phenotypes, such as cuticle defects, brittle bristles, and sterility, can be obtained. These two phenotypes are also present in *hay* (*DmXPB*) mutants (35). Therefore, we could associate these defects with deficiencies in some TFIIH functions during development. We also observed new phenotypes that we did not find in *hay* mutants. For instance, homozygous organisms died in the third-instar larval or the pupal stage; these cases were pro-

duced by heterozygous females, and the maternally contributed p52 might have sufficed to allow development until these stages. Interestingly, both larvae and pupae homozygous for *Dmp52* mutations are significantly smaller than wt organisms. In addition heteroallelic flies with the *EP3605* and mrn^1, mrn^3, or mrn^5 mutations can develop to adults but are smaller and sterile. These phenotypes, together with the fragile-bristle phenotype, the reduction of total TFIIH levels, and slow development, resemble TTD in humans and generate a minute-like phenotype in *Drosophila*. In general, minute phenotypes arise from abnormalities in protein synthesis during development, including a reduction in the ribosomal gene copy number (43); therefore, a reduction in the basal transcription levels from TFIIH mutants may have a similar effect. It is important to note that human patients affected with TTD and CS are short in stature, and therefore, there is an interesting correlation between this human phenotype and the slow-growth phenotype of the *Dmp52* gene.

"Cancer-XP-like" phenotypes in *Dmp52* mutants. Some *Dmp52* mutant larvae and pupae have melanotic tumors. These tumors are caused mainly by the overgrowth of particular tissues, generally hemocytes, that later melanize (22). Similar tumors can be found in *Drosophila* mutants affected in other kind of functions, such as transcription modulators and chromatin-remodeling factors, as well in the innate immune system and the JAK/STAT pathway (2, 50, 51), but not in *haywire* mutants (35). Furthermore, defects in DNA repair in *Drosophila*, particularly from disruption of the *Rad50* gene, which participates in double-strand repair, also generate melanotic tumors. Intriguingly, *Rad50* mutants also have cuticle defects (20). Recently, it has been reported that mutations in the damaged DNA binding protein 1 in *Drosophila*, which participates in NER and in the regulation of the innate immune response, generate chromosomal instability and melanotic tumors (50, 51).

In humans, deficiency in TFIIH activity has been associated with tumor generation, and polymorphisms in XPD are linked to different kinds of cancers (references 7 and 17 and our unpublished results). Current evidence suggests there is no casual relationship between XPD polymorphisms, reduced DNA repair, and increased cancer risk. In addition, it is very well known that patients affected with XP develop skin cancer with high frequency (30). Therefore, it is possible that deficiencies in transcription and DNA repair caused by mutations in *Dmp52* are linked to melanotic tumors. In support of this hypothesis, we observed different types and degrees of chromosomal aberrations in mitotic chromosomes in *Dmp52* mutants. The most frequent abnormalities were the presence of chromosome breaks and partial chromatid condensation at different mitotic stages. In mammals, chromosomal fragility is common in solid tumors (25), and chromosome instability has been reported in fibroblasts derived from XP individuals, as well as uncontrolled DNA breakage in UV-irradiated XPD from cells of patients (3, 5, 28). However, there are few studies of global genome integrity in patients with deficiencies in TFIIH (17). Therefore, the outcome of combinations of mutations in *Dmp52* with mutations in tumor suppressor genes, such as p53 and retinoblastoma in the fly, needs to be further investigated by conducting genetic analysis that is impossible in humans.

Pleiotropy and TFIIH mutants. *Dmp52* mutants are pleiotropic and produce different phenotypes during fly development. We have observed a similar situation in *hay* mutants (XPB) and have demonstrated that the type of mutation or the subunit affected in TFIIH affects genes differentially (21, 53). Mutations in *Dmp52* produce more diverse developmental defects than *hay* mutations. This phenomenon could be explained by two possibilities that are not mutually exclusive. The first is that mutations in the structural components of TFIIH, such as altered p52, may have a more drastic effect on complex functions, since it is not only important for TFIIH assembly, but also modulates XPB ATPase activity. Alternatively, because TFIIH interacts with multiple factors in transcription and DNA repair, a mutation in p52 may not only affect the assembly of TFIIH and its activities, but can also abolish some of these specific interactions. These two points may also explain the absence of human diseases related to HP52 mutants. That is, such mutations are probably early embryonic lethal in mammals because of the requirements for early embryonic gene transcription. However, we cannot exclude the possibility of the future discovery of a human syndrome caused by mutations in HP52.

When working with multifunctional protein complexes, such as TFIIH, pleiotropic phenotypes are difficult to interpret, because the complexes participate in so many processes. The question to ask is whether pleiotropy can be resolved by identifying the multiple functions a particular protein has within the complex. In our approach to studying TFIIH, we are close to refining the mechanisms, and we can uncover the domains of the protein that are required for each aspect of the biochemical phenotype and thus connect them to the complete organism phenotypes. Then, we can propose a working model to understand TFIIH pleiotropic defects. We think that p52 is a bifunctional protein by virtue of anchoring to the TFIIH complex and, using different domains, by binding to XPB to modulate the activity of the enzyme. Additionally, we could assign specific p52 regions as differentially modulating the NER and transcriptional activities of XPB. Because p52 is bifunctional, we predict that it will be possible to recover mutants that fully distinguish the anchoring and modulation of XPB activities. The existing mutants are a good start, because they identify functional domains, but more detailed structure-function studies are required to understand the mechanisms.

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From the beginning: the basal transcription machinery and onset of transcription in the early animal embryo

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Transcription onset in the early animal embryo is a fundamental process required for proper embryonic development. Depending on the species, transcription onset occurs at what specifically appears to be different developmental stages. However, studies in early embryos from different animal models have shown that components of the basal transcription machinery play fundamental and highly regulated roles at the onset of transcription. The state of the basal transcription machinery in the embryo seems to be equivalent in different organisms at transcription onset. The dynamic balance between putative activators and repressors as well as the chromatin/cytoplasmic ratio seem to be coordinated with basal transcription factors in order to activate zygotic transcription. Here we discuss and compare the regulation of the basal transcription machineries and their activations among early embryos of different model organisms.

Abbreviations: ZGA, Zygotic Gene Activation; MBT, Mid Blastula Transition; RNPII, RNA Polymerase II; RNPIII, RNA Polymerase III; CTD, Carboxi Terminal Domain; PIC, Pre Initiation Complex; TAF, TBP Associated Factor; TBP, TATA Binding Protein; TFII, Transcription Factor II; CMV, Cytomegalovirus; BrUTP, Bromo Uridine Triphosphate; TRF, TBP Related Factor; HAT, Histone Acetyl Transferase.

Introduction.

The transition dynamics of a transcriptionally active oocyte to a silenced one and transcription activation in the early animal embryo after fecundation is a fascinating phenomenon. It requires that the basal transcription machinery and the chromatin structure to be modulated at different levels so transcription can be tuned on in the embryo, a mechanism coordinated with the specific developmental characteristics of each animal and generally established by maternal factors [1]. Although many clues have been uncovered on how this process might be regulated, still definitive answers are elusive. Over the past 20 years, researchers have learned a great deal about the function of the transcription machinery involved in eukaryotic cells. However, signals and pathways responsible for activating these mechanisms for the first time at the beginning of animal development are still not completely understood. As we have mentioned, the earliest events in animal embryo development are controlled by maternal factors produced during oogenesis. However at specific developmental times that are characteristic of each species, Zygotic Gene Activation (ZGA) becomes fundamental for proper embryo development. Here we review and compare what is known regarding the role of the basal transcription machinery at transcription onset in embryos of four classical animal models where it has best been studied: *Drosophila, Caenorhabditis elegans, Xenopus laevis* and the mouse.

A quick overview of the basal transcription machinery for mRNA synthesis in eukaryotic cells

The saga for the identification and functional characterization of the components required for RNA polymerase II (RNPII) transcription has generated a detailed view of how this process occurs, although it is far from finished. Since in this review we discuss what it is known of the role of some RNPII transcription factors in ZGA, as initial point we will describe in general fashion, the function of some of the more relevant factors during transcription activation. However, to further deepen in this subject, we recommend two recent excellent reviews [2,3].

The Pre-Initiation-Complex. Many factors are involved in RNA synthesis in eukaryotic cells. Transcription of mRNA involves the assembly of multiple protein factors responsible for the formation of the Pre-Initiation-Complex (PIC) [2, 3, 4] (Fig.1). The principal general factors involved in assembly of the PIC are TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIH, RNPII and the Mediator. These factors, together with RNA polymerase II (RNPII), are required to initiate transcription [3]. A summary of the PIC components and its protein composition are presented in Table I.

The function of each individual factor has been experimentally determined, mostly in reconstituted *in vitro* assays [3]. TFIIA functions to stabilize binding of TFIID to the target DNA. It can also act as antirepressor and coactivator [5]. TFIIB helps determine the position of transcription initiation, stabilizes the TBP-TATA complex, it is required for the recruitment of TFIIF and selects transcription initiation site [6.7]. TFIID contains the TATA-Binding–Protein (TBP) and at least 14 TBP-Associated-Factors (TAFs), although in human cells it has been found about 20 different TAFs. These factors collectively bind DNA proximal to the transcription start site (at the TATA box motif in some promoters) and act as a platform for the incorporation of TFIIA and TFIIB. Among the components of TFIID, some TAFs show tissue-specific variants, and some animals have TBP-related factors (TLF) that have specific and in some cases equivalent functions to TBP [2, 4, 8,]. Some TAFs can also be found in other complexes different to TFIID and TBP also can be found as component of complexes involved in transcription activation by RNPI and RNPIII such as SL1 and TFIIIB respectively [9,2]. In addition, TFIID may work as coactivator and several enzymatic activities have been found like protein kinase, ubiquitin activating and conjugating activities as well as histone acetyl transferase activity. All these activities are necessary at some point in different promoters for their transcriptional activation [2,3,4]. TFIIE stabilizes the open complex, recruits TFIIH and facilitates the formation of an initiation complex allowing promoter clearance [10]. TFIIF acts in concert with other basal factors to recruit RNPII, and it is involved in the formation of the open complex [11]. TFIIF is also is involved in the recruitment of TFIIE and TFIIH. TFIIF together with TFIIB participates in the selection of the transcription initiation site and facilitates RNPII elongation [12]. TFIIH is a multifunctional complex that also participates in DNA repair and cell cycle control [13]. During transcription initiation, TFIIH facilitates the formation

of the open complex by the action of two ATP dependent helicases XPB and XPD and the Cdk7 kinase subunit that phosphorylates the RNPII large subunit Carboxy Terminal-Domain (CTD) allowing the polymerase to escape from the promoter. Recently it has also been reported that the p44 subunit of TFIIH has an ubiquitin ligase activity [14].

The RNPII large subunit CTD domain. The function of all the former mentioned factors allows RNPII to initiate and elongate transcription. RNPII is composed of 12 subunits that are highly conserved in all eukariotic cells. RNPII was first identified by Roeder and Rutter in 1969 together with RNPI and RNPIII. Five of the RNPII subunits are also present in RNPI and RNPIII [15]. The Carboxy-Terminal-Domain (CTD) of the largest RNPII subunit contains tandem repeats of the heptapeptide, YSPTSPS. The number of repeats of this amino acid sequence differs among species, with the *Drosophila* and human CTDs containing 42 and 52 repeats, respectively. The CTD may be phosphorylated at two serine residues (S2 and S5) of each repeat. It is generally accepted that a non-phosphorylated CTD is involved in PIC assembly and transcription initiation, while the hyper-phosphorylated form is required for transcription elongation. Phosphorylation and de-phosphorylation of the CTD occurs during the assembly and disassembly of the PIC, and different phosphorylation patterns are required to recruit the capping, splicing, transcription termination and 3´end processing factors during transcription. Thus, the stage of RNPII-CTD phosphorylation in a given promoter may define the transcriptional status of the associated gene [16,17].

The Mediator. Another complex that has recently has been the subject of studies about transcription initiation by RNPII is the Mediator. The Mediator is a general co-factor that transduces signals from different transcription factors to the PIC. The Mediator is composed of complexes comprising 11 to 14 different proteins in animals, or 24 in yeast. In human cells, two forms of the mediator have been identified, under different purification protocols [18]. Structural and biochemical studies have shown that the Mediator has three structural modules, namely the head, middle and tail. The head module interacts with RNPII and is thought to modulate conformational changes in RNPII. The head also interacts with specific transcriptional activators. The middle contacts RNPII at the place

where it intersects with the tail module. This interaction seems to allow conformational changes to take place in the Mediator, facilitating recruitment of the RNPII. In addition, a subgroup of 4 proteins form a specific module that some times can be identified as component of the Mediator. This module includes a Cyclin-Dependent Kinase (CDK8) along with Cyclin C, which modulates CDK8 kinase activity. CDK8 can phosphorylate Serines 2 and 5 in the CTD as well as Cyclin H (a component of TFIIH) inactivating the kinase activity present in TFIIH, suggesting that it negatively regulates the PIC. The Mediator complex that contains the CDK8 module can repress transcription in vitro and there are evidences that CDK8 may be involved in the negative regulation of specific genes. In addition, CDK8 also phosphorylates specific transcription factors having a locus specific effect [19,20]. Thus, the Mediator may act as both a negative regulator and a stimulatory cofactor for transcription. In addition to its (CDK8) kinase activity, it has been found to also have Histone-Acetyl-Transferase activity in yeast, but so far it has not been identified in other organisms [21-23]. Other multiprotein factors may also participate in general transcription. Examples of these are SAGA and SLIC complexes in yeast and the TBP-Free-TAFII-containing-Complex (TFTC) and STAGA in animals, which have multiple enzymatic activities and contain several TAFs that stimulate transcription by interacting with the PIC [for review see, 2,3].

From silencing to activation.

During oogenesis, an important transition takes place at level of gene expression, transcriptional active chromatin changes to silenced chromatin during meosis. This implies that chromatin is modified and transcription factors, in general, are excluded from the compacted chromosomes. This is even more dramatic in spermatogenesis where the whole genome is shut down and hyper-compacted. Only after fertilization and at different developmental stages, specific for each species, transcription is activated again, but now in totally undifferentiated and totipotential cell, in some cases like in the mouse transcription begins at the two pronuclei stage or in the fly after the 13th mitotic division.

The onset of transcription at ZGA. The analysis of different models has demonstrated that maternal factors are deposited in the oocyte cytoplasm with all the necessary components, including proteins and mRNA´s, necessary for the transition from silent to transcriptional active chromatin. For instance about 50% of the mRNA´s encoded in the *Drosophila* genome are present in the early embryo by maternal contribution [24] and in mice maternally deposited mRNA compose about 40% of the total genome encoded transcripts [25].

In the case of *Drosophila*, after the pro-nuclei fusion, the embryo goes through 13 synchronic nuclear divisions without cell divisions, resulting in the formation of a syncytium. However, during the first 8 divisions, only the S and M phases of the cell cycle are completed and there is no evidence of transcription. During nuclear divisions 8-10, however, a weak transcriptional wave initiates and histone, GAP, pair rule and sex determination genes are expressed. After nuclear division 13, cellularization of the embryo takes place, the cellular blastoderm forms and widespread transcription is activated in somatic cells [26] (Figure 2). The rate of mRNA transcription between the syncytial blastoderm and the cellular blastoderm are different as it has been clearly demonstrated that there is only low mRNA synthesis until the cellular blastoderm [27, 28], and that poly-A+ containing RNA transcription is dramatically increased in cellularized nuclei when compared to syncytial blastoderm nuclei [29, 28].

In the case of the early embryo development of the nematode *C. elegans*, which has asymmetrical and asynchronous cell cleavages maternal factors are segregated and then become involved in ZGA. The first signals of zygotic transcription are detectable at the four-cell stage (Fig. 2). Intriguing experiments showed that inactivation of RNPII using RNAi or α-amanitin only affected post-gastrulation transcription [30] (Fig. 2), thus indicating that maternally contributed factors mediate the development of *C. elegans* embryos until the 100-cell stage, without requiring any new transcripts.

An important point that has to be consider is that in *Drosophila* and *C. elegans* the activation of transcription in germ cells occurs later in development than in the somatic cells. Transcriptional repression in the germ cells of both organisms is maintained at the level of RNPII elongation [31]. In the case of *Drosophila* this repressed state in the germ cells requires the PCG peptide as well as Nanos and Pumillo [32]. In *C. elegans*,

transcriptional repression requires the product of the gene PIE-1and the Nanos homologues NOS-1 and NOS-2 [33]. The repressed sate in the germ cells correlates with the absence of tri-methylated lysine 4 in the histone H3 (H3K43me) in the germ cell chromatin [31], which is a mark for active chromatin, linked to RNPII elongation and suppression of cryptic transcription [34].

Xenopus has been a classical vertebrate model for the study of transcription onset in the early embryo. In 1975 Lanclos and Hamilton showed that high transcriptional activity occurred during *Xenopus* oogenesis, but transcription was inactivated at oocyte maturation [35]. Similar to post-fertilization *Drosophila* embryos, the *Xenopus* embryo undergoes 11 rapid mitotic cycles that are essentially transcription-free [36], with maternally contributed factors mediating embryonic development. Although a small amount of transcriptional activity can be detected after the sixth mitotic division in *Xenopus* embryos [36], global ZGA begins at the Mid-Blastula Transition (MBT), when the embryo is at the 4000-cell stage (Fig. 2). At this point, a shift from synchronous to asynchronous divisions takes place. Therefore, also in the case of the African frog two transcriptional waves can be postulated.

In mammals, ZGA occurs at different developmental stages depending on the species. In mouse, high transcriptional activity occurs during early oogenesis, then after germinal vesicle breakdown, the oocytes arrest at metafase II and not transcription is detected [37]. This process takes about 14 hrs; transcription can then be detected only about 10 hrs later, after fertilization and only when the pronuclei are already formed. During this time, several cytoplasmic processes take place, including the translation and systematic degradation of maternally-deposited mRNAs [38]. From the one cell stage to the two cell stage a degradation of about 90% of the stored RNA in the oocyte takes place [39]. Thus, some of the basal transcription factors or their corresponding mRNAs are maternally contributed and used to activate embryo transcription. For still unknown reasons, most of the transcription that occurs at the one cell stage happens in the male pronuclei [39] (Fig. 2). A recent review indicates that the mouse genome is activated in the embryo at the two cell stage [40], however there is evidence that the first signals of transcription, although at low levels, occur even before the fusion of the two pronuclei [38]. Therefore also in the mouse embryo, two transcriptional stages have been identified, a minor transcriptional

wave at the one-cell stage and a second major transcriptional wave at the two-cell stage [41]. This conclusion is supported by experiments that have shown that at the one cell stage there is an important RNPII dependent incorporation of BrUTP; and at two cell stage the RNA synthesis has a clear increase in BrUTP incorporation. The BrUTP incorporation in the one cell stage is only 40% of the total BrUTP incorporation at the two cell stage, again indicating two different stages of transcriptional activation. The higher levels of BrUTP incorporation acquired in the two cell stage are maintained in subsequent developmental stages [41,42]. Therefore in the mouse, the basal transcription machinery is ready to activate, after fertilization, the transcription of characteristic genes such as the ones encoding heat shock proteins, transcription factors, components of the translational machinery and factors involved in splicing [38, 41, 42].

A relatively new and useful tool for the identification of the initial genes transcribed is the use of DNA-microarrays in *Drosophila,* mice and *C. elegans,* unfortunately in *Xenopus*, only until very recently robust genomic data is available. In the case of *C. elegans*, studies analyzing lineage specific zygotic transcripts have shown that there are at least 275 genes that increase their transcription rate at the stage of 12 cells, when compared with the 4 cell stage [43]. This suggests that even if ZGA activation begins in *C. elegans* at the 4 cell stage, later in development, higher transcriptional activity ensues. With this information it can also be proposed that in *C. elegans* there are also two transcriptional waves as in the case of *Drosophila, Xenopus and mice.* The first wave at the 4 cell stage and second wave of broad transcription at the 100 cell stage, required for the continuity of embryo development (Fig. 2).

Maternal mRNA degradation and ZGA. An important point that has only been considered recently is the switch between maternal encoded mRNAs and the activation of zygotic transcripts. In *Drosophila* it has been shown that simultaneously with gene activation, maternal mRNA degradation also takes place. In the fly, maternal mRNA degradation occurs by two mRNA degradation pathways. One takes place before MBT and is maternally encoded and some of the maternal mRNA 3' UTR's contain cis-acting elements required for this degradation pathway. The second mechanism is activated just before MBT. Both mechanisms are essential for embryo development [44]. Also in the fly,

about 20% of these transcripts degradation is dependent of the multi-translational regulator SMAUG, which is modulated by cell cycle regulators like PAN GU [24]. On the other hand a recent elegant study in *Drosophila* using chromosome ablation and microarrays demonstrates that that expression of some of the first zygotic transcripts correlates with the concomitant maternal mRNA degradation. The zygotic transcripts are especially enriched in transcription factors that modulate the subsequent differentiation program. Interestingly, the earlier zygotic transcripts come from intron-less genes that are regulated by an specific transcription factor that enhance its expression at ZGA [45]. Based in these results, De Renzis et al., [45] postulate that the absence of introns in the first genes that are transcribed have an evolutionary advantage that facilitates the fast mitotic divisions of the 10-13 stages. These results demonstrate a coordinated relation between ZGA and maternal mRNA steady state levels and suggest the role of a specific activator (we will discuss later the role of repressors and activators). The analysis of how this activator is regulated will be an interesting challenge for future experiments.

The mice early embryo has also been the subject of study in order to know what are the first genes transcribed at ZGA. Two recent reports that use microarrays compare the transcript profile of one cell to two cell embryos treated with or without α-amanitin reported intriguing results. In one case, mRNA transcripts sensitive to α-amanitin in the one cell stage were not found [46]. In the second only one transcript sensitive to α-amanitin was detected [47]. A possible explanation is that in the α-amanitin treated embryos, BrUTP incorporation at the one cell stage is due to the transcription of poly A minus RNPII dependent RNAs like snRNA, soRNAs and histone transcripts. It is also possible that in the methods used, mRNA´s with very short poly A+ tails could not detected. Based on these results it can be speculated that the novo transcription (RNPII dependent BrUTP-RNA incorporation) at the one cell stage embryo only includes a small fraction of the total mature mRNA´s detected in the microarray experiments. It is likely that most of them are maternal transcripts that encode for components used in mRNA metabolism and protein synthesis that are required for the embryo to continue and maintain development from the one cell to the two cell stage, but not further, since about 90 % of the oocyte stored RNA are degraded by the end of the 2 cell stage [42,48] and posterior developmental stages [47].

Recently the role of microRNA (miRNA) in the transition from maternal to zygotic mRNA has been the focus of attention. For instance, in early zebrafish embryos, the degradation of some maternal mRNAs is controlled by a microRNA that is expressed at the ZGA. This microRNA also promotes deadenylation of target mRNAs, suggesting that this pathway may have some control the change from maternal to zygotic transcripts in the early embryo [49]. During mouse oogenesis miRNAs are expressed and stored in the mature oocyte. Indeed Dicer is required for oocyte maturation [50]. Intriguingly about the 60% of the maternal miRNAs are degraded between the one cell to two cell embryo [51], an stage in which most of the maternal mRNAs are also degraded. Later, at the four cell stage, miRNA levels rise again. These interesting results suggest a dynamic role of the miRNA machinery at the transition from maternal to zygotic transcripts and it is supported by the finding that a Dicer deficient embryos were not able to develop from the one cell to the two cell stage [51]. Therefore, the miRNA and RNAi machineries may have a role in the transition from maternal to zygotic mRNA in the early animal embryo.

Although these studies identify the transition from maternal to zygotic RNA it is important to point out that not only maternal RNA is stored in the early embryo, but also proteins, that in many cases function in early and late developmental stages. This is the case of homozygous lethal mutants in several basal transcription factors in *Drosophila,* in which the protein stored in the oocyte allows all the embryo development until larval stages [52,53]. Related to this point some maternal transcripts and their translated protein products are present and act to ensure transcription during the first cell division in *mice*. One of these, Cyclin A2, modulates the kinase activity of CDK2 (CycA2-CDK2), as shown by studies revealing that inactivation of Cyclin A2 inhibited transcriptional activation at the one-cell stage [54]. Although the CycA2-CDK2-specific transcription activating targets are not yet known, it seems possible that this kinase activity is required to activate transcription factors that may include members of the basal transcription machinery.

PIC players and CTD phosphorylation at Zygotic Gene Activation.

A number of researchers have sought to analyze ZGA in different models by identifying the components and dynamics of the basal transcription machinery and its influence in CTD phosphoryaltion. Immunostaining, RNAi and protein ablation have been

used in early embryos to answer how the PIC formation is regulated at the transcription onset.

Nuclear translocation of PIC components. Different studies have collectively shown that the subunits of RNPII, as well as other components of the basal transcription machinery, are maternally contributed to the cytoplasm of the early embryo and are translocated from the cytoplasm to the nuclei just before ZGA. For example in *Drosophila*, the RNAPII large subunit can be detected in the nuclei of the syncytial blastoderm at mitotic cycle 7, but its phosphorylated active form is detected only at the cellular blastoderm stage, which correlates with the high transcriptional activity seen at this stage [55]. Also in the fly, TBP may be initially detected in the nuclei at mitotic cycle 8, just before expression of the first zygotic genes [56]. On the other hand TFIIH, which is also deposited in the early embryo cytoplasm by maternal contribution, can be detected in the cytoplasm of the syncytial blastoderm, before the onset of transcription [57]. However, it isn't until after nuclear division 9 that the core and CAK of TFIIH are translocated into the nuclei, where they are positioned at active gene promoters for ZGA [57].

As in other animals, the mouse oocyte contains large amounts of RNPII; the levels of phosphorylated CTD decrease during oocyte maturation, and increase after fertilization at the end of the one-cell stage, showing good correlation with ZGA [58]. Interestingly, a transitory phosphorylated form of the large RNPII subunit may be detected prior to zygotic transcription [58]. Most of the transitory phosphorylated form is cytoplasmic, while the hypo-phosphorylated form is present both in the cytoplasm and the nucleus and the persistent form of hyper-phosphorylated RNPII is mostly nuclear. Translocation of RNPII from the cytoplasm to the nuclei takes place at the late one-cell stage, about 9h after fertilization and preferentially in the male pronucleus. This nuclear translocation does not require the *de novo* synthesis of RNA or proteins, and therefore follows a program established during oocyte development. Based on these results, it seems logical to believe that the nuclear translocation of RNPII and its CTD phosphorylation are a major level of developmental regulation for ZGA in the mouse embryo [58]. In addition, components of the TFIID factor like TBP and TAF1 are practically undetectable in the pronuclei after fertilization. TBP can be identified inside the male mouse pronuclei after 4hr post

fertilization and TAF1 after 6 h. Therefore, the nuclear localization of TBP and TAF1 together with the RNPII translocation from the cytoplasm to the nuclei correlates with ZGA. The evidence observed in *Drosophila* and mice suggests that activation of the basal transcription machinery is a limiting step for ZGA [59,60]. However, these experiments cannot distinguish if the regulated translocation of the basal transcription machinery causes transcription onset or because of transcriptional activation the basal transcription components accumulate in the early embryo nuclei.

TAF's and the mediator in CTD phosphoryaltion. *C. elegans* is probably the organism in which more studies on the role and dynamics of the basal transcription machinery at ZGA have been made. The CTD-phosphorylated RNPII form can be detected in the somatic nuclei at the 4 cell stage. On the other hand the relation between CTD-phosphorylation and some TAFs has been documented. For example, TAF5 can be identified in the nuclei at the two-cell stage, whereas TAF10 and TAF11 are present in four-cell stage nuclei [61]. By the time gastrulation occurs, all three of these maternally-derived TAFs are located inside the nuclei. RNAi inactivation of these three TAFs and TFIIB showed that Ser 5 CTD phosphorylation was reduced by TFIIB RNAi, only slightly affected by TAF5 RNAi, and not affected by TAF10 and TAF11 RNAi, while Ser-2 phosphorylation was largely reduced by inactivation of TFIIB or TAF5 and partially reduced by inactivation of TAF10 and TAF11 [61]. These results indicate that Ser-5 phosphorylation, which is critical to open complex formation, requires TFIIB and has some need for TAF5, but not TAF11 and TAF10, whereas Ser 2 phosphorylation, which is required for RNPII-based elongation, requires TAF5 and TFIIB and has some need for TAF10 and TAF11. On the other hand, TAF5 is required for general gene expression, while TAF10 and TAF11 are only involved in the expression of a fraction of genes at these stages. This may correlate with the observation that TAF5 can be found inside nuclei at the two-cell stage, while TAF10 and TAF11 do not show nuclear localization until the four-cell stage. In future experiments, it could be interesting to determine if the early nuclear localization of TAF-5 is related to its broad role in transcription. Intriguingly, the TAF10 and TAF11transcriptionally-associated genes were found to be metazoan-specific genes

[50], suggesting that loci specific functions may be conferred to different TFIID-related complexes during ZGA.

In addition, studies using RNAi against TAF1 and TAF2 in early embryos have shown that both proteins are required for mRNA transcription of several genes in early *C. elegans* embryos, furthermore the total RNPII-CTD phosphorylation levels were dramatically reduced as consequence of the RNAi knock-down [62]. Altogether these studies indicate that an important component for transcription activation in the *C. elegans* embryo is TFIID, although it seems that there are TAFs that are dispensable for the expression of some genes at ZGA while others are not. On the other hand, the demonstration that TFIID specific TAFs like TAF1 and TAF2 are necessary for gene expression at ZGA in *C. elegans*, at least for most of the genes that have been analyzed is intriguing, since it has been demonstrated that these proteins are not required for the expression of about 86% and 97% of the yeast genes respectively [63]. This implies that the complexity of the ZGA in metazoans requires the ordered action of TFIID including the sequential use of most of its TAFs.

A recent study in *Xenopus* on a new TBP-related factor named TBP2, that is only present in vertebrates, shows that in some genes it has redundant roles with TBP during ZGA in regulating their expression, but not in others [64]. By performing knock -down and ChIP experiments it was demonstrated that TBP2 is positioned in promoters of genes expressed in early embryos, in particular at the elongation factor 1a promoter. TBP2 is required for the transcription of a subset of genes in the early embryo and can substitute the role of TBP in some promoters, but it is restricted to early development. Therefore, different TBPs may have various functions in controlling different genes at the point of ZGA.

On the other hand, both in the mouse embryo as in *Xenopus*, it seems that some TBP related factors can substitute or have a redundant TBP function for the expression of some genes in early development. Homozygous early embryos that have been knock-out for the TBP gene have active transcription by RNPII [65]. In these embryos, the TBP related factor 3 (TRF3) appears to be one of the redundant factors involved in RNPII transcription at ZGA. TRF3 is expressed in oocytes and early embryos and ChIP experiments demonstrated that although there is selectivity for some promoters between

TBP and TRF3, depending on their relative amount these factors can be redundant for the activation of some promoters at ZGA [65]. However, in these TBP-KO experiments it can not be discarded the presence of enough TBP deposited in the egg by maternal contribution that may mask the TBP requirements in the early embryo.

In *C. elegans*, the CTD- phosphorylation at Ser 2 and Ser 5 is affected when the highly conserved RGR-1 subunit of the Mediator complex is knocked down [66]. Therefore, the connection that has been reported between the Mediator activities and CTD phosphorylation in yeast is also a key element in ZGA in *C. elegans* [66]. In addition, a TBP-like factor (CeTLF) was found to be located in nuclei at the two-cell stage and its inactivation by RNAi prevent CTD phosphorylation and the expression of specific early patterning genes. The fact that it is present in similar amounts with TBP, suggest that has a specific function in the activation of specific genes at ZGA [67,68].

CTD Kinases. Studies into the kinases responsible for phosphorylating the CTD have identified the involvement of CDK9, which together with Cyclin T forms the p-TEFb complex and phosphorylates Ser 2 of the CTD [69]. It has been proposed that this modification coordinates the capping and elongation of RNPII transcripts. Notably, pTEFb is essential for the expression of early embryonic genes and the phosphorylation of Ser 2 in the CTD, and the elongation factor SPT-5. Experiments in *C.elegans* have shown that p-TEFb and SPT-4/SPT-5 have opposing functions during RNPII elongation, and P-TEFb is thought to mediate several different post-initiation pathways [69]. However, it is not yet known how P-TEFb functions in these differential pathways. In addition, the role of P-TEFb in early gene expression has not yet been analyzed in the four-cell *C. elegans* embryo, when ZGA occurs.

Another kinase identified as being involved in CTD phosphorylation is CDK7, which phosphorylates CTD Ser 5 and is a component of TFIIH [13, 70, 71]. Since zygotic transcripts are not required in the early *C. elegans* embryo until the 100-cell stage, a conditional mutant and RNAi were used to determine the roles of CDK7 in embryonic transcription and cell cycle control. Interestingly, CDK7 inactivation at the 50-cell stage led to developmental arrest, demonstrating that CDK7 plays a role in cell cycle control. Similar results have been found in *Drosophila* either using Cdk7 mutants or by the microinjection

of early embryos with anti-CDK7 antibodies [57, 72-74]. In addition, a conditional CDK7 mutant showed changes in the early expression patterns of the zygotic genes, reduced CTD phosphorylation and a longer cell cycle [73, 74]. Thus, in the early *C. elegans* and in *Drosophila* embryos, CDK7 appears to participate in both cell cycle control and transcription onset [72-76]. Future studies will be required to determine how CDK7 simultaneously and differentially participates in both functions at the same time.

Based in the analysis of the dynamics of the CTD phosphorylation during the *C. elegans* oocyte silencing and its subsequent transcription activation in the embryo Walker and colleagues [77], proposed a very attractive model that suggests that transcription activation is prepared during oocyte maturation. This model is based in experiments that show that RNAi inhibition of the CTD phosphatase Fcp-1 in oocytes that have entered diakinesis, which are inactive in transcription, accumulate large amounts of CTD Ser 5 phosphorylated in the nuclei. Intriguingly, the RNAi inhibition of components in the ubiquitination pathways, produce the same effect. In addition, the RNAi inhibition of Cdk7 and other components of PIC, together with the inhibition of Fcp-1 do not show CTD-Ser-5 phosphorylation, suggesting that the CTD phosphorylation occurs in the context of the PIC. Thus this model propose that at the beginning of diakinesis the oocytes initiates and maintains transcriptional silencing by the regulation the of basal transcription machinery having abortive transcription process, but it is already positioned at selected gene promoters that require rapid activation at the ZGA. Supporting this hypothesis is the fact that when oocyte maturation is stimulated there is also an increase in the CTD phosphorylated at Ser5. Other important point that this work contributes is that the balance and regulation between CTD phosphatases and CTD kinases plays a major role in ZGA. However, other components of the PIC are required for the CTD phosphorylation and it has not been demonstrated that the RNPII phosphorylated and the rest of the basal transcription machinery are positioned in chromatin that is going to be transcribed at ZGA, this will be a major task since the system is highly dynamic.

In addition, substantial evidence indicates that CTD phosphorylation CTD is a key aspect in the initiation of mRNA synthesis in *Xenopus* [78]. After fertilization, the CTD is rapidly dephosphorylated in the *Xenopus* embryo. This un-phosphorylated state is maintained through out several divisions, and then the CTD is rapidly phosphorylated at the

Mid Blastula Transition [79]. Although the dynamics and roles of the TFIIH and P-TEFb complexes have not yet been analyzed in *Xenopus*, experiments in other models have shown that these factors play fundamental roles in CTD phosphorylation. Additional work will be required to examine the mechanisms governing post-fertilization CTD dephosphorylation in *Xenopus* and other organisms.

Taking together the accumulated knowledge about the dynamics and functions of PIC components at ZGA it is clear that PIC formation is a key element for the transition of a silent to an active genome. On the other hand the CTD phosphorylation is an important mark that determines the transcription activation state in the animal embryo. However, techniques with more resolution, such as chromatin immunoprecipitation, will be required to identify the PIC status in specific genes at ZGA.

Repressors, activators and the Chromatin/cytoplasm ratio.

The regulation of the PIC components to activate transcription has to be coordinated with the modulators that selectively activate gene expression. One of the more interesting hypothesis on transcription activation in early embryo development has been the influence of repressors and activators. In general, these ideas come form studies in which the introduction of exogenous DNA accelerate ZGA, probably by the titration of a global repressor as well as the fact that some activators are necessary for the transcriptional activation of specialized genes. In initial studies, specific exogenous DNA templates capable of being assembled as nucleosomes were injected into *Xenopus* embryos, in an effort to determine at what point in development they were transcribed [80]. These experiments revealed that templates carrying RNA polymerase III (RNPIII) promoters were transcribed prior to the MBT, and further suggested that their transcription was activated by a shift in the ratio between chromatin and transcription factors [80]. An interpretation of this model suggests that as cell division progresses, increased DNA assembly into chromatin titrates a repressor (i.e. a global repressor, histones or some other structural chromatin factor), allowing transcription of the newly relaxed chromatin.

This hypothesis was challenged by Almouzni and Wolfe, who demonstrated that the introduction of an exogenous DNA template containing a RNPII promoter (CMV), was only transcribed after MBT [81]. Interestingly, when they simultaneously injected their

promoter construct along with a non-specific DNA (e.g. λ DNA), they observed histone titration but no transcription from the RNPII promoter. However, they observed transcription following co-injection of template DNA along with non-specific DNA and TBP. They also corroborated that the activation of RNPIII promoters can occur before MBT in the absence of TBP, but showed that the RNPII promoter required TBP [81]. To complement these experiments, the authors used the minimal adenovirus E4 promoter under the control of the yeast GAL4 transcriptional activator and found that introduction of exogenous GAL4 activated transcription of the exogenous DNA template prior to the MBT. Based on these observations, it was proposed that activation of transcription in the early *Xenopus* embryo is not only due to the DNA-based titration of repressors, but is also mediated by the limiting activity of transcription factors, such as TBP [81]. This interesting hypothesis seems to explain the observed results, and may indicate that TBP acts as a limiting factor for activating transcription at the MBT in the *Xenopus* system. However, activation of the minimal adenovirus E4 promoter by GAL4 did not require extra TBP under their experimental conditions.

Supporting the hypothesis that TBP may be a limiting factor, another study showed that TBP protein levels are minimal in the early embryo and increase prior to the MBT, when ZGA begins [82]. Therefore, besides the DNA/chromatin ratio, the levels of active components of the basal transcription machinery (e.g. TBP) are also important for mediating transcription activation at the MBT. Interestingly, TBP is essential for continued development after gastrulation, and a TBP-like factor (TLF/TRF2) is essential for continued development after the MBT [83]. However, it was also shown that transcription is repressed before MBT, even if a transcriptional activator is positioned at its DNA binding element; this seems to indicate that the activator is unable to recruit the basal transcription machinery at this developmental stage. After MBT, the histone/DNA ratio approaches its normal somatic value and the PIC can be assembled [84]. Therefore, it can be concluded that the ratio of transcriptional activators and repressed chromatin is a major determinant for ZGA in *Xenopus*.

Interestingly, early studies showed that ZGA is not initiated simultaneously in all nuclei of the *Drosophila* embryo [85]. For example, the *fushi taratzu* gene (*ftz*) is initially transcribed in some nuclei at mitotic cycles 8-9, with *ftz* transcription seen in increasing

number of nuclei during later rounds of division. It has been shown that, the ratio between the number of nuclei and the concentration of a repressing transcription factor is critical for triggering zygotic transcription in *Drosophila*. This is the case for *ftz*, which is repressed by *tramtrack*. It has been proposed that as the nuclei/cytoplasm ratio increases across sequential nuclear divisions, a titration effect stochastically decreases the effective concentration of the transcriptional repressor in each nucleus, allowing gradual transcription activation [85]. This model is highly attractive if we assume that all the genes activated at the point of ZGA have a titratable repressor which may be specific or not. Since it is obvious that many genes do not have a known specific repressor, and this model implies that the repressor binds to the control region of genes, it seems alternatively possible that a global repressing chromatin structure exists in the earliest embryo and eventually becomes titrated through successive nuclear divisions, thus obviating the need of specific repressor factors

In *Drosophila*, some of the genes encoding the earliest zygotic transcripts (e.g. those involved in sex determination and patterning) share a DNA motif called the TAGteam. This motif is required for their expression at mitotic cycles 8-10, suggesting the action of an activator [86]. Interestingly, in the previously mentioned work in *Drosophila* by De Renzis et al., [45] a similar element was identified upstream of the gene promoter encoding the early transcribed genes, This cis-motif is of about 7 base pairs and is recognized by the Bicoid Stability Factor (BSF), that was previously identified as factor that binds the *bicoid* mRNA. BSF can activate transcription in early embryos in constructs containing this 7 bp motif upstream of reporter gene, demonstrating that it operates at ZGA. This discovery is highly relevant, since for the first time it is demonstrated the existence of a general activator for ZGA. Since putative BSF homologues are present in other organisms, it will be imperative to know if BSF has equivalent function in early vertebrate embryos.

Taking into account the abovementioned repressor and activators activity, it has been proposed that the balance between repressors and activators may determine the early expression of some genes [86]. However, while these are important arguments for the regulation of genes expressed in specific cells of the early embryo, additional work will be required to determine how these processes are coordinated with the basal transcription machinery. In addition, it is important to note that in the fly most of the genes activated in

the syncytial nuclei at mitotic cycle 10 continue to be expressed during and after cellularization, suggesting that establishment of molecular memory around some promoters allows continued transcription. Interestingly, TFIID is maintained in active promoters during mitosis, and the presence of maternal BSF [45] in the different early fly embryo stages, suggest that a memory mark may be established [87].

To make the process a little more complex in the fly, the overexpression in *Drosophila* embryos of the Nanos (Nos) protein, which is a translational repressor of *Hunchback* mRNA, required for the establishment of the posterior embryo development, appears to have a global repressing role in transcription by reducing the CTD-phosphorylation levels in the early somatic cells [88]. This data suggests that Nos may inhibit the translation of factors required for CTD phosphorylation in the posterior embryo, thus maintaining reduced levels of global transcription that may be required for posterior development. However, which factors involved in CTD-phosphorylation are regulated by Nos are unknown. Some of the possible Nos targets could be mRNA´s encoding different TFIIH subunits. However, it has been demonstrated that different TFIIH protein subunits are already present in the early embryo by maternal contribution [57]. Therefore, it is also possible that the Nos targets may be mRNA´s encoding other components of the basal transcription machinery, required for RNPII activation and as consequence a reduction in the phosphorylated CTD levels. Thus in *Drosophila* Nos not only participate in the regulation of ZGA in the germ cells, but also in the somatic cells.

An intriguing hypothesis making a link between maternal mRNA degradation and activators like BSF that bind both RNA and DNA, could be that the activator is titrated by maternal RNA in the early embryo and that when it becomes degraded, the activator is released and thus allowed to activate transcription. A simple experiment to test this hypothesis should be to enhance maternal mRNA degradation and thus analyze whether ZGA becomes accelerated under these conditions.

Chromatin and epigenetic marks at transcription onset

Transcriptional activation in the embryo requires additional processes beyond the coordinated activation of the basal transcription machinery. For instance, epigenetic changes occur in chromatin during the transition from a transcriptionally silent to

transcriptionally active genome. During early *Xenopus* development, DNA methylation is required for transcriptional silencing before MBT, and inactivation of maternal DNA methyltransferases was shown to cause transcriptional activation to occur two cell divisions earlier than normal [89]. In addition, the promoters of genes that are activated at the MBT, such as TFIIA and c-Myc, show reduced methylation at this stage, whereas repetitive DNA elements remain methylated [90]. These data collectively indicate that DNA methylation plays an important role in the onset of transcription in the early embryo.

Global chromatin changes at the onset of transcription have been observed in early *Drosophila*. During the first nuclear divisions, the condensed chromatin preferentially contains the high mobility group protein, HMG-D, but not the H1 histone. In contrast, H1 accumulates after nuclear division 7, when the nuclei become more compact and transcription is activated [91]. Therefore, a correlation exists between the HMG-D/H1 and nuclei/cytoplasm ratios at the point of ZGA. It has been speculated that this change in chromatin composition is needed because more condensed chromatin is required to permit the next nuclear divisions. However, during these stages the chromatin structure around the activated genes must be relaxed, or at least in a configuration that allows activators and the basal transcription machinery to access their promoters.

The heterochromatin domains in the chromosomes of the *Drosophila* embryo, seems to be established early in development. A recently discovered Histone H3-lysine 4 demethylase encoded for the suppressor of variegation gene Su(var)3-3 as component of a complex with the Histone H3-lysine 9 methylase Su(var)3-9, coordinate the demethylation of lysine 4 and methyaltion of lysine 9 in histone H3, therefore, participating in the determination of the limits between heterochromatin and euchromatin during cellularization [92]. Therefore, at the same time of transcription activation, the epigenetic boundaries between transcribed and silenced chromatin have to be established (Fig 3).

Chromatin rearrangements, nucleosome modifications and changes in protein composition also have been shown to occur before zygotic transcription in mammals [93]. It is intriguing that the male pronucleus is transcriptionally active before the female pronuclei. To achieve this, male pronuclei suffer a drastically chromatin remodeling process including the change from a protamines for histones that are maternally supplied. During this process histone modifications changes seems to be required to activate

transcription [92]. For instance, in the one cell mice embryo, there are differences in the amount of histone H3-lysine-9 methylation between the male and female pronuclei. Contrary to the female pronuclei, the male pronucleus has practically no H3-Lys-9 methylation and its H3-Lys-4 methylation is increased, correlating with its higher transcriptional activity [94, 95]. Later at the four cell stage the H3-lysine-9 methylation is similar between the two parental genomes. In addition histone acetylation is increased in the male pronuclei and at the same time reduced in the female one. This process occurs by the action of histone acetyl transferases and de-acetylases supplied by the oocyte [94, 95]. A recent study using a combination of techniques including Cre-loxP gene targeting and RNAi demonstrated that the SWI/SNF2 remodeling complex subunit, BRG1 (a homologue of the Brahma protein in *Drosophila*), was required for activation of zygotic transcription in mice [96]. The transcribed genes comprised cell cycle regulators, transcription factors and components of the RNA processing machineries, and inactivation of BRG1 led to embryonic arrest at the two-cell stage. Reduced BRG1 was concomitant with reduced methylation of Lys-4 in histone H3, which marks transcriptionally active chromatin. No global reduction in histone acetylation was detected, but only a few acetylation markers were tested. A more detailed analysis will be required to determine if reduced BRG1 activity affects other histone modifications. Based on these results, a model has been proposed in which the BRG-containing SWI/SNF chromatin remodeling complex acts together with Histone Acetyl Transferases (HATs) to methylate K4 in histone H3 and activate transcription via the PIC [96]. This model of ZGA is attractive, but does not yet account for coordination with other chromatin remodeling factors and the basal transcription machinery. Indeed, there is evidence that other factors participate with the chromatin remodeling machinery at ZGA in mice. One of these factor is the Transcription intermediary factor 1α (TIF-1α) is required for the proper chromatin localization of BRG1, SNF2H and the RNPII [97]. TIF-1α has been identified as a transcription regulator and it can be associated with chromatin remodeling factors [98]. TIF-1α ablation using RNAi affects the expression of genes that are activated just after ZGA in the mouse one cell embryo. These genes include proteins that may be involved in RNA processing [97]. It is intriguing why in the microarray assay studies for the identification of α-amanitin sensitive mRNA´s at the one cell stage, none of these transcripts were identified [47, 48].

Conclusions and perspectives.

Even though ZGA occurs at different developmental stages in various species, it seems that the factors required for transcription activation, including components of the basal transcription machinery (e.g. RNPII), chromatin remodeling factors and histone modification complexes, are in general deposited during oogenesis. In *Drosophila* and *Xenopus* and perhaps other organisms, unknown factors may govern ZGA by regulating translocation of maternally-derived factors from the cytoplasm to the nuclei. Another commonality is that CTD phosphorylation and its subsequent effects on transcription are central cues that determine the basal transcription factors status in ZGA in all animals. A paradox is that in *Drosophila*, CTD phosphorylation is not detected at the mitotic cycles 8-10 in the early embryo, when the first zygotic genes are transcribed. However, since only few genes are transcribed at this point, it is possible that the immunostaining experiments have not been sensitive enough to detect only the few RNPII large subunit molecules that are actually phosphorylated at the CTD. We think that this is probably the case, since together with the RNPII, CDK7 is positioned at the promoters of the genes to be transcribed at mitotic cycles 8-10 [53].

The four organisms discussed here seem to have two transcriptional waves during ZGA. In the first wave, transcription of a selected few genes occurs early in development (at the one-cell stage in mice, mitotic division rounds 8-10 in the fly, the four-cell stage in *C. elegans* and the sixth mitotic division in the frog); these genes are likely required for the continuing of cell division and/or for the activation of the second transcription wave, which involves the expression of a larger number of genes (Figs. 2, 3). Although the possibility that the higher levels of transcription in the second wave are consequence of a general and continuous increase of transcription during development can not be discarded, the finding in *Drosophila* of gene activator specific for the early stages supports the two wave hypothesis.

Although various studies suggest that DNA/chromatin and/or DNA/repressor ratios control transcription activation during early embryonic development, the generality of this model is not yet fully accepted in all animal models. Future work will be required to assess the participation of activators for transcription of large gene sets, as well as the

coordination of repressors and activators with the basal transcription machinery and various chromatin remodeling factors (Figure 3).

The more we learn about the onset of transcription in different animal models, the more similar the pathways appear. The observed differences like the different transcriptional activation timings, seem largely superficial, and probably reflect an escalation effect caused by differences in cytoplasm volume and the chromatin/cytoplasm ratio. For example, we have to take in account that in the fly, the activator BSF is produced during oogenesis, and only is active until mitotic division 10. When this ratio is low at the time of fertilization, more synchronous replication cycles will be required to trigger induction of global and space/time-regulated somatic transcription. To address this possibility, it would be interesting to carefully study ZGA in organisms that have very small chromatin/cytoplasm ratios, such as chicken and zebra fish. Another possible basis for the observed differences in transcription onset among species could be evolutionary adaptation of the basal transcription machinery to various developmental conditions and/or the development of species-specific transcription factors (activators and repressors) responsible for coordinating the balance between transcriptionally permissive or repressive chromatin.

It will also be valuable to study how factors that activate ZGA at the correct time are coordinated with the basal transcription machinery, and how chromatin structure and its regulation determines the activation of a somatic nucleus introduced into an early embryo [99]. The later experiments may help us understand why so few cloned organisms develop to maturity, and may be of primary importance for future progress in somatic cell cloning, de-differentiation and tissue engineering. Therefore, new studies using updated genomic approaches (e.g. ChIP on chip and new imaging techniques) will be useful for investigating early transcription requirements at the genome-wide level, as well as for identifying and characterizing the roles of global repressors, activators and components of the basal transcription machinery during ZGA.

Before ZGA some maternal mRNA´s are degraded while others can remain until late developmental stages, particularly in *Drosophila*. Therefore, the mechanisms involved in the selectivity of mRNA degradation and the possible role of microRNA and RNAi machineries is just beginning to be explored. On the other hand we know very little about

which are the genes that are differentially transcribed in the two transcriptional waves at ZGA. Therefore similar microarray experiments to the ones performed in one cell and two cells mice embryos, in the presence and absence of α-amanitin will be highly relevant if they are integrated with ChIP on chip experiments.

In summary, from the information discussed here, three models are compatible with the available data that can be proposed: 1) There are general transcription repressors and activators that are deposited during ovogenesis either as mRNA or as proteins and they translated or postraductionally modified in an orderly way during early development to activate gene transcription. 2) There are general transcription repressor or repressors that are simply titrated as the chromatin/cytoplasmic ratio diminishes. 3) Components of the basal transcription machinery are deposited in the egg cytoplasm, their translocation into the nuclei is then tightly regulated in order to start zygotic transcription. It is important to stress that these possible mechanisms are not excluding and some form of all of them may be involved in ZGA. These models open several exiting new questions. For instance: How is regulated the nuclear translocation of the PIC components before ZGA? How cell cycle and ZGA are coordinated? What is the role of chromosome condensation on gene expression during early nuclear divisions? How the activator, BSF initiates ZGA? There is a mechanism that links the RNAi machinery with maternal mRNA degradation and P-bodies formation? Thus the problem of how ZGA is achieved in the early animal embryo still requires many answers and is far to be resolved. However with the all accumulated knowledge in these four model organisms and the viability of new techniques, make this time ripe to look forward for the solution of this problem.

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Table I. Components of the RNPII Pre-Initiation-Complex (PIC).

Factor	No. Subunits	Functions.
TFIIA	3	Stabilize TBP-TATA complex; Co-activator and anti-repressor.
TFIIB	1	Binds TBP; Selection of the transcription Initiation site; Binds RNPII
TFIID	TBP TAF´s (around 14)	Promoter binding factor. Recognition of promoter elements; Histone-Acetyl-Transferase activity; Ubiquitin activating and conjugating activities.
TFIIE	2	Formation of the initiation complex; Interacts with TFIIH; Facilitates RNPII promoter escape.
TFIIF	2	Binds RNPII; Recruits TFIIE and TFIIH; Participates in RNPII promoter escape and elongation.
TFIIH	10	Allows RNPII promoter escape and elongation; Helicase, ATPase, Kinase and Ubiquitin ligase activities. Also involved in DNA repair and cell cycle control
RNPII	12	Transcription initiation, elongation and termination; CTD can be phosphorylated ubiquinated and glycosilated.
Mediator	12-24	Positive and negative modulator; Transduce signals from different transcription factors to the PIC.

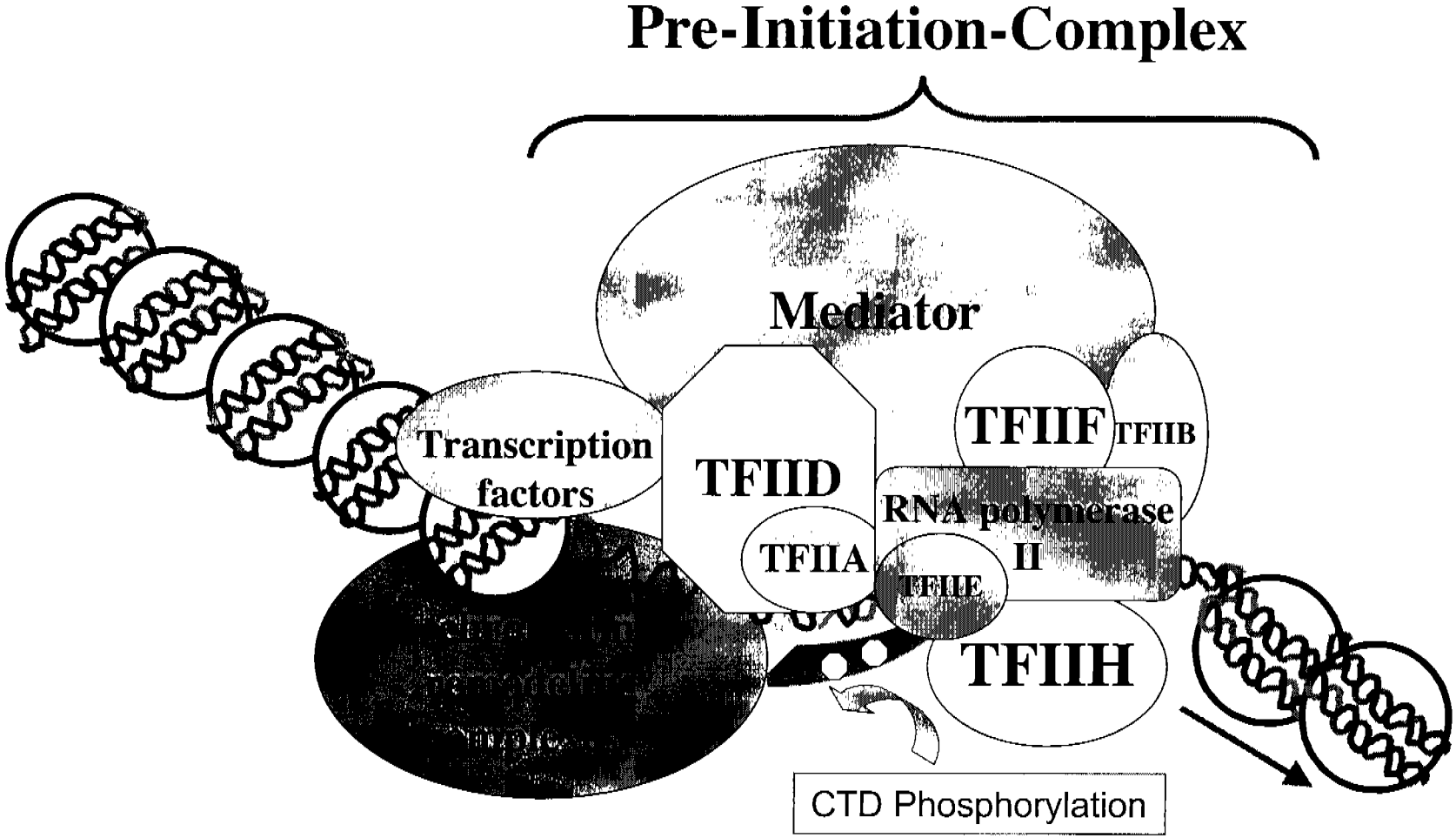
Figure legends:

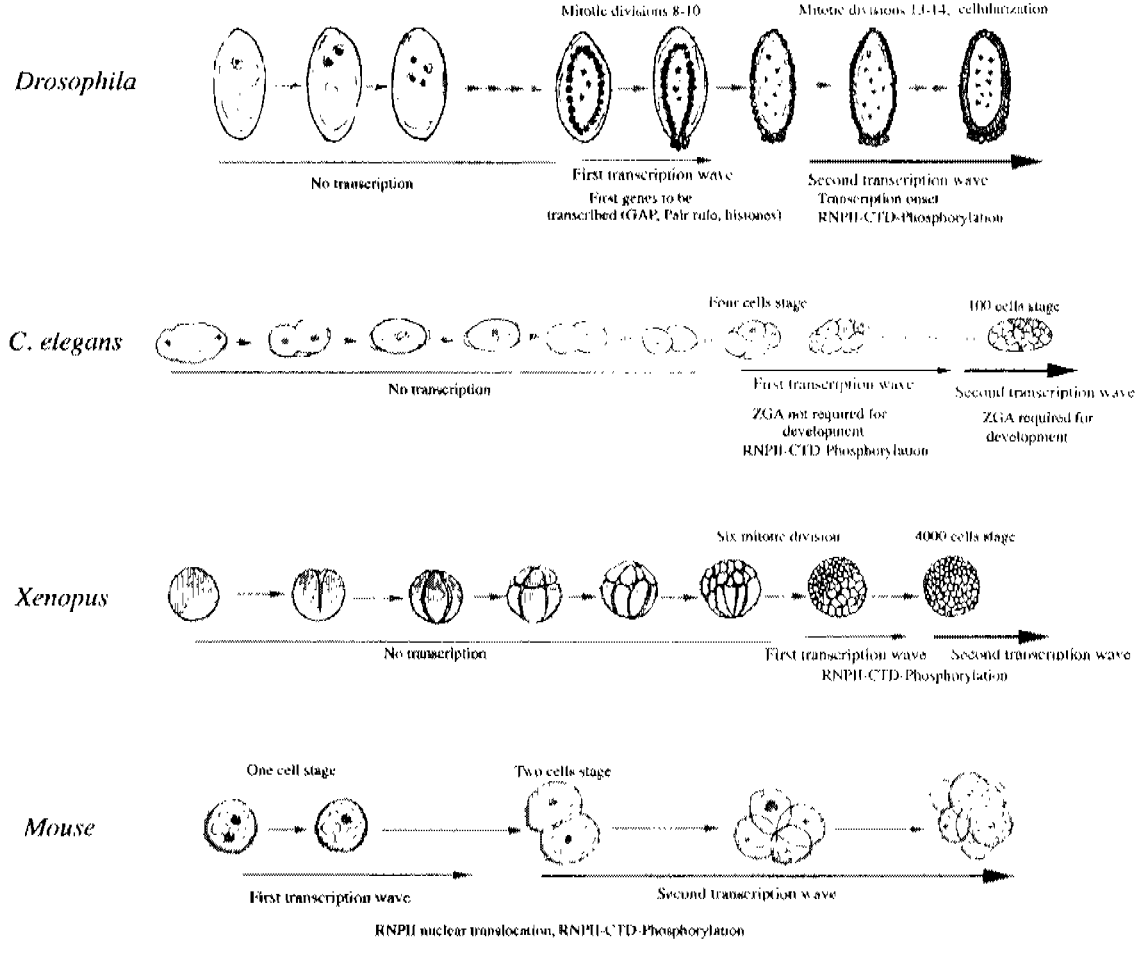
Fig. 1. **Schematic representation of the RNA polymerase II Pre-Initiation complex (PIC).** Based on different experimental conditions, two models have been proposed for assembly of the PIC on class-two gene promoters (RNPII promoters). In the first model, the different basal factors are sequentially assembled on the promoter. The second postulates the existence of an RNPII holoenzyme containing most of the basal factors except TFIID, with promoter recognition by TFIID triggering recruitment of the holoenzyme to the promoter. The figure represents the PIC at the moment of the open complex, when a bubble is formed in the DNA and the RNPII large subunit CTD is phosphorylated. In brief, the components and functions of PIC are: TFIIA blocks TAF1 transcription inhibition and also may participate as positive and negative regulator. TFIIB is important to define the transcription initiation site. TFIID helps the binding of TFIIB and RNPII and is the target on repressors and activators. TFIIF stabilize the binding of RNPII with the promoter and is important for the open complex formation. TFIIE stabilize the open complex and interacts with TFIIH. TFIIH is fundamental for the melting of the DNA around the transcription initiation site and phosphorylates the RNPII-large subunit CTD domain. The Mediator binds the RNPII and acts as transducer of activators and repressors to the PIC. For more details consult the text and Table I. Tissue specific transcription factors and chromatin remodeling complexes are not considered to be part of the PIC.

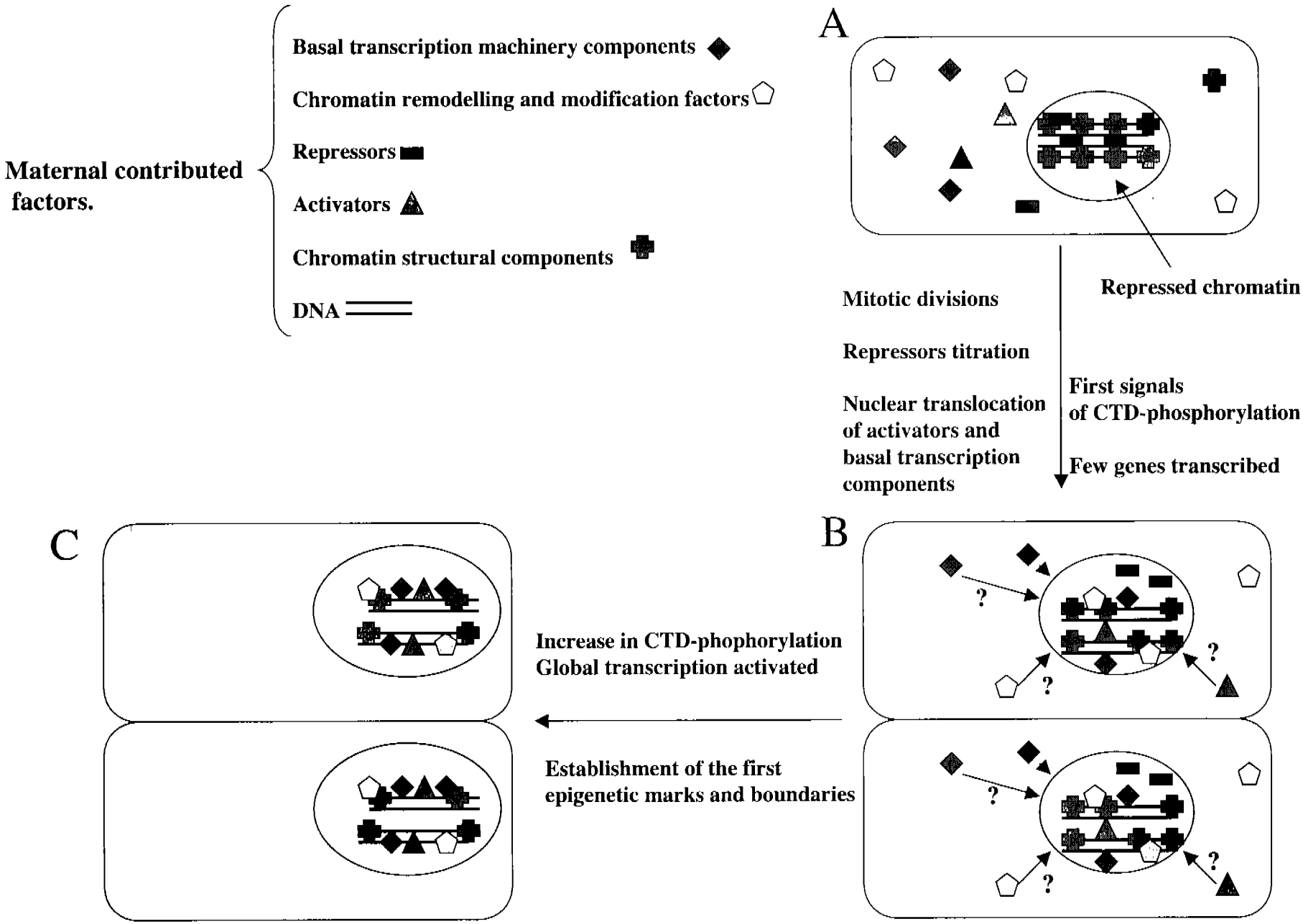
Fig. 2. **Comparative diagram of transcription onset in *Drosophila, C. elegans, Xenopus* and mouse during the early developmental stages**. In the case of the fly, *C. elegans* and *Xenopus*, the initial mitotic divisions occur without transcription. In the mouse embryo, transcription initiates at the one-cell stage, at the male pronuclei, in a process that requires several hours. In all organisms seems to have two transcriptional waves. The first one only involves few selected genes, and the second establish somatic gene expression.

Fig. 3. **General mechanisms for transcription onset in animal embryos**. A) Maternal factors required for the activation of transcription are deposited in the egg cytoplasm, both in form of mRNA and protein. These factors include components of the basal transcription machinery, chromatin remodeling and modification factors, transcriptional repressors,

transcription activators and chromatin structural components (histones and other chromatin proteins). At this stage, no transcriptional activity is detected and the chromatin is in a repressed stage. B) During the first stage of transcription activation, specific repressors and structural chromatin components are titrated by nuclear division, while transcription activators and components of the basal transcription machinery (e.g. including RNPII) are translocated into the nuclei. In some cases, such as in *Drosophila*, the basal factors translocate at various nuclear division stages, but are all present inside the nuclei at the time of transcription onset, when the CTD is phosphorylated and transcription elongation takes place at a few specific zygotic genes. C), Later in development and in some organisms at MBT, global transcription is activated, correlating with increased CTD phosphorylation levels. At this stage, the first epigenetic marks are established.


Pre-Initiation-Complex
Mediator
Transcription factors
TFIID
TFIIA
TFIIF
TFIIB
RNA polymerase II
TFIIE
TFIIH
CTD Phosphorylation


Drosophila
Mitotic divisions 8-10
Mitotic divisions 11-14, cellularization
No transcription
First transcription wave
First genes to be transcribed (GAF, Pair rule, histones)
Second transcription wave
Transcription onset
RNPII-CTD-Phosphorylation
C. elegans
Four cells stage
100 cells stage
No transcription
First transcription wave
ZGA not required for development
RNPII-CTD Phosphorylation
Second transcription wave
ZGA required for development
Xenopus
Six mitotic division
4000 cells stage
No transcription
First transcription wave
RNPII-CTD-Phosphorylation
Second transcription wave
Mouse
One cell stage
Two cells stage
First transcription wave
Second transcription wave
RNPII nuclear translocation, RNPII-CTD-Phosphorylation


Maternal contributed factors.
Basal transcription machinery components
Chromatin remodelling and modification factors
Repressors
Activators
Chromatin structural components
DNA
A
Repressed chromatin
Mitotic divisions
Repressors titration
Nuclear translocation of activators and basal transcription components
First signals of CTD-phosphorylation
Few genes transcribed
B
?
?
?
?
?
?
Establishment of the first epigenetic marks and boundaries
Increase in CTD-phophorylation
Global transcription activated
C