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A Mammalian RNA Polymerase II Holoenzyme Containing All Components Required for Promoter-Specific Transcription Initiation

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Summary

The protein kinase MO15/CDK7 has recently been shown to be associated with the general transcription factor TFIIH and to be capable of phosphorylating the RNA polymerase II carboxy-terminal domain. Here, we show that a monoclonal MO15/CDK7 antibody coimmunoprecipitates, from a rat liver nuclear extract, all components of the RNA polymerase II transcription apparatus required for initiation at the albumin and adenovirus major late promoters. The immunoprecipitate includes RNA polymerase II, TFIID, TFIIB, TFIIH, TFIIF, and TFIIE, but is devoid of transcriptional activator proteins, such as HNF1, HNF4, and C/EBPα. The finding of an autonomously initiating RNA polymerase II holoenzyme in mammalian cells suggests conceptual similarities between transcription initiation in prokaryotes and eukaryotes.

Introduction

Transcription initiation can be divided into three major steps: initiation complex assembly, isomerization, and promoter clearance (McClure, 1980; Knaus and Bujard, 1990; Eick et al., 1994; Goodrich and Tjian, 1994). In the first step, RNA polymerase and ancillary proteins bind reversibly to promoter DNA to form the closed complex. In the second and nearly irreversible step, a short stretch of DNA around the transcription initiation site becomes unwound and serves as a template for abortive transcription, the synthesis of short oligoribonucleotides. Finally, in the third step, the RNA polymerase leaves the promoter to elongate the primary transcript.

In Escherichia coli, closed complex assembly consists of the binding to the promoter of the RNA polymerase holoenzyme, composed of the initiation factor σ and the four core enzyme subunits $(\alpha)_2$, β′, and β. After isomerization and during promoter escape, σ dissociates from the elongating core enzyme. The efficiency of each of the three initiation steps can be subject to regulation by transcriptional activator or repressor proteins binding to specific operator sequences in the proximity of the promoter. Moreover, the same regulatory protein can affect different initiation steps, depending on the precise promoter context. For example, the catabolite activator protein (CAP) enhances closed complex formation at the *lac* promoter, stimulates isomerization at the *gal* promoter, and facilitates promoter escape at the *malT* promoter (Menendez et al., 1987; for reviews see Kolb et al., 1993; Busby and Ebright, 1994).

For protein-encoding genes of eukaryotic cells, transcription initiation involves over 40 polypeptides. These include the 12 subunits of RNA polymerase II (pol II), the general transcription factors (GTFs) TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIH, and TFIIJ (for reviews see Roeder, 1991; Maldonado and Reinberg, 1995), and, at least in yeast, a number of additional proteins called SRBs (for suppressors of RNA polymerase B) (Koleske et al., 1992; Thompson et al., 1993; Liao et al., 1995). Most of the GTFs are themselves composed of multiple subunits. For instance, TFIID, a particularly important component in promoter recognition, consists of the TATA box–binding protein (TBP) and at least eight TBP-associated factors, called TAFs (Dynlacht et al., 1991; Kokubo et al., 1994). Based on in vitro reconstitution studies with purified components, initiation complex assembly has originally been proposed to occur in multiple discrete steps. In this model, TFIID, TFIIA, and TFIIB associate with the promoter to form the DAB preinitiation complex, before pol II and the remaining GTFs associate to complete the initiation complex (for reviews see Roeder, 1991; Corden, 1993; Maldonado and Reinberg, 1995). Transcriptional activator or repressor proteins may mediate their effect through protein–protein interactions with GTFs, such as TBP (Truant et al., 1993), TAFs (Goodrich et al., 1993; Gill et al., 1994; Chiang and Roeder, 1995), TFIIB (Roberts and Green, 1994; Kim and Roeder, 1994), and TFIIH (Xiao et al., 1994; Drapkin and Reinberg, 1994).

A novel perspective on eukaryotic transcription has recently been opened through the discovery of a holoenzyme in yeast (Koleske and Young, 1994; Kim et al., 1994; for review see Emili and Ingles, 1995). The largest pol II subunit harbors a carboxy-terminal domain (CTD) composed of 26 (yeast) to 52 (mammals) repeats of the heptapeptide YSPTSPS or minor permutations thereof (Corden, 1990; Young, 1991). Elegant genetic studies by Young and coworkers (Koleske et al., 1992; Thompson et al., 1993; Liao et al., 1995) have identified the CTD as a target for interaction with new components of the pol II transcription machinery, SRBs. These novel proteins were found to be associated with a large complex, containing pol II, TFIIB, TFIIF, TFIIH (Koleske and Young, 1994; Kim et al., 1994), and, depending on the purification procedure, TBP (Thompson et al., 1993). When complemented with TBP and TFIIE, this large complex, named holoenzyme, was capable of efficient and accurate in vitro transcription (Koleske and Young, 1994).

The serines of the CTD are targets for extensive phosphorylation by a number of protein kinases (for review see Corden, 1993). One of these CTD kinases is associated

with human TFIIH and has recently been identified as MO15/CDK7 (Roy et al., 1994; Serizawa et al., 1995; Shiekhattar et al., 1995). A structurally related kinase, KIN28, is similarly associated with yeast TFIIH (Feaver et al., 1994). Biochemical evidence suggests that CTD phosphorylation may be required for promoter clearance. First, the CTD of elongating but not "free" pol II is highly phosphorylated (Corden, 1993; Wuarin and Schibler, 1994), and second, unphosphorylated, but not phosphorylated, CTD binds avidly to TBP (Usheva et al., 1992). However, successful in vitro transcription can be accomplished without CTD phosphorylation (Serizawa et al., 1993), opening room for other speculations.

Given the high evolutionary conservation of components of the pol II transcription apparatus, it is likely that the holoenzyme is a general feature of eukaryotic cells. Yet, the existence of such a complex has not yet been reported in mammalian cells. By using a monoclonal MO15/CDK7 kinase antibody (Tassan et al., 1994), we were able to immunoprecipitate a pol II holoenzyme from rat liver nuclear extracts. The immunopurified complex contains all of the components required for efficient and promoter-specific initiation on linear and circular templates.

Results

The CTD Kinase MO15/CDK7 Is Associated with a Pol II Holoenzyme

MO15/CDK7, in conjunction with cyclin H and an additional protein (p36), has originally been described as a cyclin-dependent kinase (CDK)-activating kinase (Fesquet et al., 1993; Fisher and Morgan, 1994; Solomon et al., 1993; Poon et al., 1993; Tassan et al., 1994; Mäkelä et al., 1994). Recently, the same MO15/CDK7 kinase complex has also been identified as the TFIIH-associated CTD kinase (Roy et al., 1994; Serizawa et al., 1995; Shiekhattar et al., 1995). This feature makes this kinase a likely candidate for a regulator of transcription by pol II. Since MO15/CDK7 kinase has been studied mostly in rapidly dividing cells, we examined whether it also accumulates in nonproliferating, terminally differentiated cells, such as rat parenchymal hepatocytes. Immunoblot analysis of transcriptionally competent rat liver nuclear extracts with the monoclonal antibody MO-1.1 revealed the presence of MO15/CDK7 (Figure 1B, lane 3). We then used the MO-1.1 antibody to immunoprecipitate MO15/CDK7, to examine whether TFIIH subunits and perhaps other components of the pol II machinery would be associated with this kinase. To this end, MO-1.1 antibodies were adsorbed to paramagnetic beads impregnated with goat anti-mouse antibodies, and the resulting beads were incubated with a cleared liver nuclear lysate. The beads were then concentrated by magnetic attraction and washed three times extensively with a large excess of binding buffer containing the nonionic detergent Triton X-100. The proteins present in the immunoprecipitate (IP) were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and subjected to Western blot analysis with various antibodies against general components of the pol II transcrip-

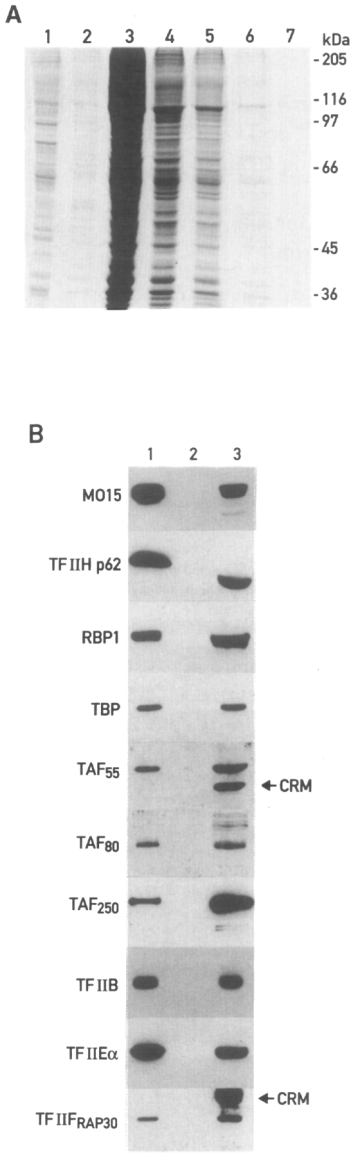

Figure 1. A Monoclonal MO15 Kinase Antibody Immunoprecipitates a Complex Containing Several Components of the Pol II Transcription Machinery

MO15 kinase, and proteins associated with it, were immunoprecipitated with the monoclonal antibody MO-1.1 from liver nuclear extract and analyzed by SDS–PAGE, Coomassie blue staining (A), and immunoblotting (B). As a negative control, immunoprecipitation was conducted in parallel with an equal amount of an irrelevant monoclonal antibody (anti-NANP).

(A) Coomassie blue–stained proteins. Lane 1, MO-1.1-immunoprecipitated proteins from 400 μg (50 μl) of nuclear extract (NE); lane 2, anti-NANP immunoprecipitated proteins from 400 μg (50 μl) of nuclear extract; lane 3, 80 μg of NE; lane 4, 20 μg of NE; lane 5, 5 μg of NE; lane 6, 1.25 μg of NE; lane 7, 0.4 μg of NE.

(B) Western blots. Lanes 1 and 2, immunoprecipitated proteins recovered from 200 μg (25 μl) of nuclear extract with the monoclonal antibodies MO-1.1 and anti-NANP, respectively; lane 3, 40 μg of NE. Antibodies used in the Western blots are indicated at left. Nuclear proteins were separated on 10% polyacrylamide gels, except for RBP1 and TAF_{250} immunoblots, for which 7% polyacrylamide gels were used. Note that in the immunoblots with nuclear extracts the TAF_{55} and $TFIIF_{RAP30}$ antibodies strongly cross-react with unrelated proteins (CRM for cross-reactive material) that disappear during immunoprecipitation with the MO-1.1 antibody.

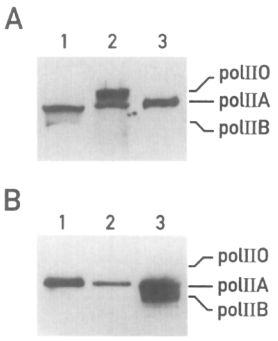

Figure 2. MO-1.1 Antibody Coimmunoprecipitates Only the IIA Isoform of Pol II

(A) A nuclear extract (lane 1), an SDS nuclear lysate (lane 2), and an MO-1.1 IP (lane 3) were displayed on a 7% SDS–polyacrylamide gel and probed by Western blotting with a monoclonal CTD antibody. The positions of the three forms of the largest pol II subunit, RPB1, are indicated.

(B) A nuclear extract, particularly rich in pol IIB (lane 3), was used in immunoprecipitations with MO-1.1 (lane 1) and anti-CTD (lane 2) antibodies. The proteins were analyzed as described in (A).

tion apparatus. As a negative control, immunoprecipitation with an unrelated monoclonal antibody, recognizing multimers of the tetrapeptide NANP, was performed. From the Coomassie blue–stained gel shown in Figure 1A, we estimated that less than 0.4% of the input nuclear lysate bound nonspecifically to the magnetic beads. As expected, the MO15/CDK7 IP contained more than 50% of the MO15/CDK7 kinase present in the input rat liver nuclear lysate (Figure 1B, lanes 1 and 3). In accordance with the association of MO15/CDK7 with TFIIH suggested by previous reports, the IP was also enriched in p62 (Fischer et al., 1992), a subunit of TFIIH (Figure 1B, lane 1). Remarkably, however, the MO-1.1 antibody also coimmunoprecipitated a sizable proportion of other essential pol II transcription components, such as the largest polymerase subunit, RPB1, and the GTFs TBP, TAF_{250}, TAF_{80}, TAF_{55}, TFIIB, $TFIIF_{RAP30}$, and TFIIEα (Figure 1B, lane 1). None of these constituents are enriched with the magnetic beads

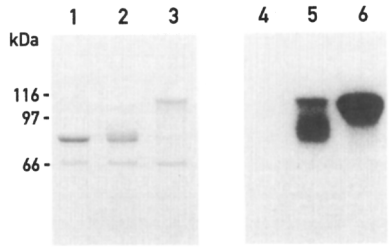

Figure 3. CTD Kinase Activity in the MO-1.1 IP

Portions (from 5 μl of nuclear extract) of the MO-1.1 (lanes 2, 3, 5, and 6) and NANP (lanes 1 and 4) IPs were incubated with 10 μCi [γ-^{32}P]ATP, 15 μM cold ATP, and a GST–CTD fusion protein (about 100 nM). After 20 min, the kinase reactions shown in lanes 1, 2, 4, and 5 were stopped by the addition of SDS–PAGE loading buffer. The kinase reactions shown in lanes 3 and 6 were continued for 20 min in the presence of 5 mM cold ATP. The proteins were fractionated by SDS–PAGE, stained by Coomassie blue (lanes 1, 2, and 3) or exposed for autoradiography (lanes 4, 5, and 6; for 20 s).

carrying the control monoclonal antibody (Figure 1B, lane 2). One concern with the immunoprecipitation of a large multisubunit complex like the one described here is that DNA-binding components, such as TFIID and pol II subunits, could be artificially tethered together by contaminating DNA. However, as will be shown below, this does not seem to be the case.

Three pol II RPB1 forms with different electrophoretic mobilities have been described: pol IIA, pol IIB, and pol IIO (for reviews see Corden, 1990; Young, 1991). Pol IIO bears a highly phosphorylated CTD and is predominantly associated with elongating ternary transcription complexes (Wuarin and Schibler, 1994; for review see Corden, 1993). Pol IIA is not or only partially phosphorylated and is the major form observed in soluble nuclear extracts. This is believed to be the initiation-competent form of pol II. Pol IIB lacks part of its CTD and may thus be a partial proteolytic degradation product of either pol IIA or pol IIO (for review see Dahmus, 1994). The proportion of pol IIB is somewhat variable in different nuclear lysates (see below), further suggesting that it may be the result of artifactual degradation. Figure 2A shows that both pol IIO and pol IIA are present in an SDS lysate of entire liver nuclei (lane 2). The transcription competent extract, obtained by extraction of liver nuclei with 0.4 M NaCl, is devoid of highly phosphorylated pol IIO, but contains pol IIA and somewhat variable amounts of pol IIB (Figure 2A, lane 1; Figure 2B, lane 3). Interestingly, pol IIA is the only polymerase in the MO-1.1 IP (Figure 2A, lane 3; Figure 2B, lane 1), suggesting that an intact CTD is required for the association of the MO15/CDK7 kinase complex with the polymerase. For the experiment shown in Figure 2B, we deliberately chose a nuclear extract in which pol IIB was relatively abundant (lane 3). This extract was also used for an immunoprecipitation with a monoclonal anti-CTD antibody. As shown in Figure 2B (lane 2), this antibody selectively precipitated pol IIA, although in the Western blot it still recognized the residual CTD associated with pol IIB (Figure 2B, lane 3). Thus, in the context of unfractionated nuclear extract, only the carboxy-terminal portion of the CTD may be accessible. Surprisingly, the MO-1.1 antibody yielded a higher proportion of the pol IIA present in the nuclear extract than the CTD antibody. We cannot discern whether this reflects differences in the affinities of the two antibodies to their respective epitopes, or whether the CTD in the large complex is poorly accessible to the antibody.

An interesting but yet unresolved question concerns the regulation of CTD phosphorylation by MO15/CDK7. If this kinase is closely associated with the CTD, why does it not convert pol IIA into pol IIO? One possibility would be that the CTD kinase only becomes activated when the holoenzyme is bound to the promoter. At least in vitro, this does not seem to be the case. The immunoprecipitated MO15/CDK7 kinase is highly active on an exogenously added glutathione S-transferase (GST)–CTD fusion protein (Figure 3), as well as on the CTD of the coimmunoprecipitated pol II (data not shown). To examine whether phosphorylation on the multiple CTD repeats occurs cooperatively, [γ-^{32}P]ATP and cold ATP were added at a relatively low

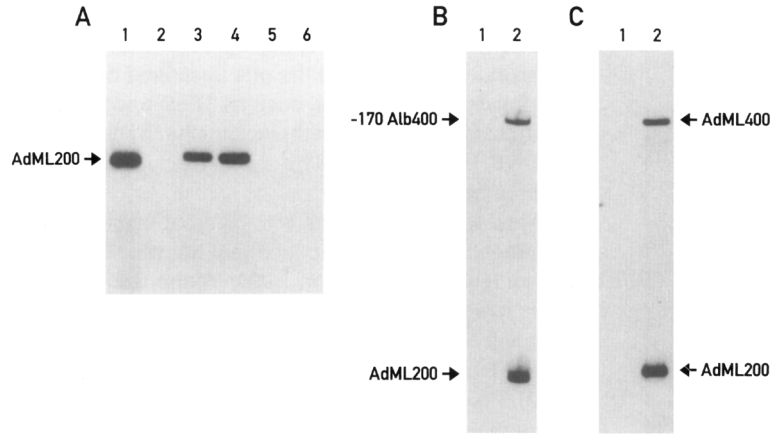

Figure 4. In Vitro Transcription with the Immunopurified Pol II Holoenzyme

(A) A supercoiled circular G-free cassette template containing the adenovirus major late promoter (AdML200, −400 to +10) was incubated with the following: lane 1, MO-1.1-immunoprecipitated proteins from 400 μg (50 μl) of nuclear extract (NE); lane 2, anti-NANP immunoprecipitated proteins from 400 μg (50 μl) of nuclear extract; lane 3, 80 μg of NE; lane 4, 20 μg of NE; lane 5, 5 μg of NE; lane 6, 1.25 μg of NE. (B) Two supercoiled circular G-free cassette templates containing either the adenovirus major late promoter (AdML200, −400 to +10) or the albumin promoter (−170Alb400, −170 to +22) were incubated with immunoprecipitated proteins from 400 μg of nuclear extract. Lane 1, anti-NANP IP (control); lane 2, MO-1.1 IP. (C) A linear G-free cassette template, AdML400, and a supercoiled circular G-free cassette template, AdML200, both containing adenovirus major late promoter sequences encompassing nucleotides −400 to +10 were incubated with immunoprecipitated proteins from 400 μg of nuclear extract. Lane 1, anti-NANP IP (control); lane 2, MO-1.1 IP.

total concentration of 15 μM in a phosphorylation reaction with 100 nM of GST–CTD fusion protein. Given that the CTD contains approximately 100 serine residues that can potentially be phosphorylated, this kinase reaction was performed at a ratio of approximately 0.75 phosphoacceptor sites per phosphate donor ATP. Consistent with this ratio, phosphorylation of the GST–CTD fusion protein did not affect the mobility of the bulk protein significantly (Figure 3, lane 2). In contrast, autoradiography of the same gel revealed that an appreciable proportion of radiolabeled GST–CTD protein shifted to lower mobilities (Figure 3, lane 5). In fact, some of these phosphorylated GST–CTD molecules reached the position observed in the kinase reaction in which the ATP concentration was increased to 5 mM, after the initial labeling at 10 nM (Figure 3, lanes 3 and 6). This observation indicates that an already phosphorylated CTD is a better substrate for further phosphorylation than an unphosphorylated CTD. Given that CTD phosphorylation can take place in the absence of promoter DNA, kinetic parameters are likely to be responsible for the lack of CTD phosphorylation in pol IIA (see Discussion).

The Immunopurified Holoenzyme Contains All Components Required for Promoter-Specific Transcription

Having demonstrated the existence of a mammalian pol II complex containing many essential GTFs, we wondered whether this complex was capable of accurate transcription initiation, similar to the E. coli RNA polymerase holoenzyme. Therefore, we incubated the MO-1.1 (Figure 4A, lane 1) and NANP (Figure 4A, lane 2) immunoprecipitated proteins and the same dilutions of nuclear extract shown in Figure 1A (Figure 4, lanes 3–6) with a G-free cassette template harboring the adenovirus major late promoter, along with the three nucleoside triphosphates ATP, CTP, and [α-^{32}P]UTP. Figure 4A demonstrates that this template is actively transcribed by the immunopurified pol II holoenzyme (lane 1). As expected on the basis of the immunoblotting results described in Figure 1B, no transcripts can be discerned in the transcription reaction with the control IP (Figure 4A, lane 2). With 80 μg and 20 μg of nuclear proteins, similar amounts of in vitro transcripts are observed as with the immunopurified enzyme (Figure 4A, lanes 3 and 4). However, no transcripts can be discerned with 5 μg or 1.25 μg of nuclear proteins (Figure 4A, lanes 5 and 6). On the basis of Figure 1A, we estimate that the magnetic beads are contaminated by no more than 1.5 μg (0.4% of input proteins), an insufficient amount for in vitro transcription (see above). Thus, the strong signal obtained with the immunopurified nuclear proteins (Figure 5, lane 1) cannot be accounted for by fortuitous contamination of the magnetic beads by components of the pol II machinery.

We also wanted to test the ability of the immunopurified holoenzyme to transcribe from the promoter of a cellular gene. Therefore, the IPs were incubated with a G-free cassette template harboring the albumin promoter (Gorski et al., 1986), in addition to the adenovirus major late template. Figure 4B shows that these two promoters are efficiently transcribed, although the adenovirus major late promoter appears to be somewhat more active.

Since our in vitro transcriptions were performed with circular supercoiled templates and since negative supercoiling has been described to facilitate transcription initiation (Parvin and Sharp, 1993; Goodrich and Tjian, 1994), we wished to determine whether linear templates are also transcribed by the immunoprecipitated complex. To this end, a circular and a linearized adenovirus late promoter plasmid were provided as templates for the immunoenriched complex. As shown in Figure 4C, the holoenzyme initiates accurately at both promoters, although the circular template produces about 3-fold more transcripts than the linear plasmid.

An issue that had to be addressed is whether components of the holoenzyme may be tethered together by DNA present in the nuclear extract, and if so, whether such interactions are required for the coimmunoprecipitation with the MO-1.1 antibody. The IP from 0.5 mg of nuclear proteins did not contain nucleic acids in amounts detectable by ethidium bromide staining after agarose gel elec-

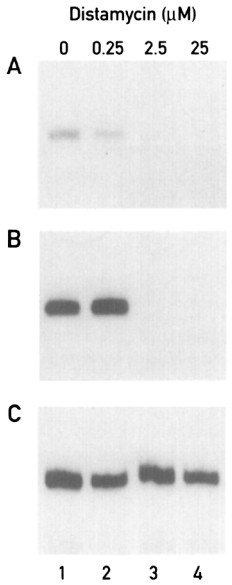

Figure 5. The Pol II Holoenzyme Can Be Purified in the Presence of Distamycin

(A) Electrophoretic mobility shift assays of human recombinant TBP (2 ng/10 μl reaction) and a double-stranded oligonucleotide spanning the AdML TATA box (4 ng/10 μl reaction) in the presence (lanes 2–4) and absence (lane 1) of distamycin. Only the polyacrylamide gel region containing the protein–DNA complex is shown.

(B) In vitro transcription from the AdML promoter in crude nuclear extracts in the presence (lanes 2–4) and absence (lane 1) of distamycin. In vitro transcriptions were performed as the one shown in Figure 4A, lane 3.

(C) In vitro transcription from the AdML promoter with pol II holoenzyme immunopurified in the presence (lanes 2–4) and absence (lane 1) of distamycin. The IPs were washed with buffer lacking distamycin before they were used for in vitro transcriptions. In vitro transcriptions were performed as the one shown in Figure 4A, lane 1.

trophoresis (data not shown). However, short heterogeneous DNA fragments, present at stoichiometric amounts with coimmunoprecipitated TBP, would probably have escaped detection by this method. We thus designed a more sensitive strategy to examine whether DNA is involved in the coimmunopurification of TBP. Distamycin, a drug with high affinity to AT-rich sequences in the minor groove of DNA, strongly competes with TBP for DNA binding (Chiang et al., 1994). As shown in Figure 5A (lanes 3 and 4), distamycin, at concentrations of 2.5 μM and 25 μM, severely interferes with the binding of TBP to a double-stranded oligonucleotide encompassing the TATA box of the adenovirus major late promoter. Consistent with this observation, in vitro transcription from the adenovirus major late promoter in liver nuclear extracts is completely abolished at distamycin concentrations of 2.5 or 25 μM (Figure 5B, lanes 3 and 4). Yet, the same drug has little, if any, effect on the immunoselection of the pol II holoenzyme with the MO-1.1 antibody, even when present at 25 μM. As demonstrated in Figure 5C, similar levels of adenovirus major late transcripts were synthesized by the pol II holoenzyme, irrespective of whether distamycin was included during its immunopurification (lanes 2–4) or not (lane 1). Therefore, DNA is unlikely to tie TFIID to the multisubunit complex described here. However, whether DNA is involved in the assembly, stabilization, or both of pol II holoenzyme in vivo remains an open question.

In conclusion, the results described in this section demonstrate that the rat liver pol II holoenzyme described here is capable of accurate in vitro transcription of linear and circular templates, and from two distinct promoters. Thus, the immunopurified mammalian holoenzyme contains all basal constituents for initiation complex assembly, isomerization, and promoter clearance.

Sequence-Specific Transcription Factors Do Not Copurify with the Holoenzyme

The activation domains of several transcriptional regulatory proteins have been shown to establish specific protein–protein interactions with various components of the pol II transcriptional machinery. Among the targets for such interactions are TBP (Truant et al., 1993), TAFs (Goodrich et al., 1993; Gill et al., 1994; Chiang and Roeder, 1995), TFIIB (Kim and Roeder, 1994; Roberts and Green, 1994), and TFIIH. (Xiao et al., 1994). Since, as shown here, all of these components appear to be part of the mammalian pol II holoenzyme, we wondered whether DNA-binding transcriptional regulatory proteins copurify with the holoenzyme. We thus examined the MO-1.1 immunoprecipitated proteins for the presence of the three liver-enriched transcription factors HNF1 (Lichtsteiner and Schibler, 1989), HNF4 (Tian and Schibler, 1991), and C/EBPα (Landschulz et al., 1989). The data displayed in Figure 6A demonstrate that none of the three transcription factors could be detected in the IP. Thus, either the three examined transcriptional activator proteins are present in a large excess over the holoenzyme, or the affinity between transcriptional activators and components of the holoenzyme are insufficient to withstand the immunoprecipitation, or both statements are true. If the holoenzyme is not associated with transcriptional activators, it should transcribe equally well from a core promoter, devoid of *cis*-acting upstream elements, and a promoter carrying such binding elements for activators. We chose the albumin promoter to test this conjecture. This promoter has been extensively studied in vitro (Gorski et al., 1986; Lichtsteiner at al., 1987; Maire et al., 1989) and in vivo (Heard et al., 1987) and has been demonstrated to be composed of the core promoter and six upstream elements, A to F, binding liver-enriched and ubiquitous transcription factors. Two such factors, HNF1 and C/EBP, occupying sites B and D, respectively, are particularly important for liver-specific albumin in vitro transcription. Two G-free cassette templates, −59Alb400 and −170Alb220, have been constructed that contain different lengths of albumin 5′-flanking sequences. The truncated promoter of the template −59Alb400 lacks both the HNF1 and C/EBP recognition sequences. Not surprisingly, this promoter is much less active than the full-length promoter of −170Alb220 in in vitro transcription assays with a crude liver nuclear extract (Figure 6B, lane 1). In contrast, and in accordance with the immunoblot data presented in Figure 6A, the upstream albumin promoter region has little impact on the in vitro

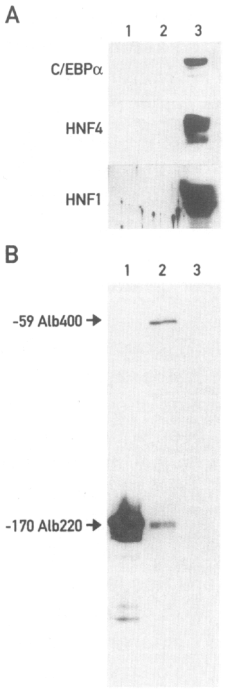

Figure 6. The Pol II Holoenzyme Initiates Transcription in the Absence of Transcriptional Activator Proteins

(A) Immunoprecipitates from 200 μg of nuclear extract were examined in Western blot experiments for the presence of the transcriptional activator proteins C/EBPα, HNF4, and HNF1: 10% SDS–polyacrylamide gels were used for C/EBPα and HNF4; 7% SDS–polyacrylamide gels were used for HNF1. Lanes 1, MO-1.1 IPs; lanes 2, anti-NANP IPs; lanes 3, 40 μg of nuclear extract.

(B) Two circular albumin promoter G-free cassette templates that do (−170Alb220) or do not contain (−59Alb400) upstream binding sites for sequence-specific transcription factors were in vitro transcribed with liver nuclear extract (lane 1), MO-1.1 immunoprecipitated proteins from 400 μg of nuclear extract (lane 2), or anti-NANP immunoprecipitated proteins (lane 3).

transcription with the immunopurified pol II holoenzyme (Figure 6B, lane 2).

The results presented in this section suggest that the holoenzyme is not associated with significant levels of transcriptional activator proteins and, hence, is not stimulated by the presence of upstream promoter elements.

Discussion

We report here the successful isolation of a pol II holoenzyme from a rat liver nuclear extract, by using a monoclonal antibody against the MO15/CDK7 kinase. This enzyme contains all of the components required for accurate transcription initiation and elongation. Our immunoblotting data suggest that a significant fraction of each, RPB1 (the largest pol II subunit), TBP, TAF_{250}, TAF_{80}, TAF_{50}, TFIIB, TFIIH (MO15/CDK7), $TFIIF_{RAP30}$, and TFIIE (subunit α), are immunoprecipitated with the MO-1.1 antibody. We estimated that, in intact nuclei, these proteins are about 30-fold more concentrated than in the nuclear extract used

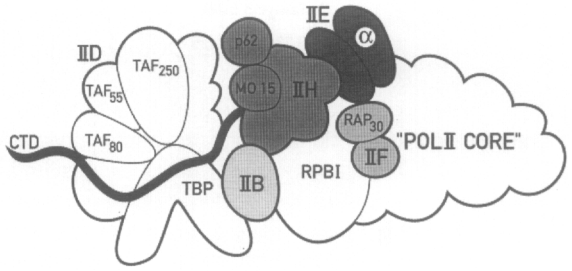

Figure 7. Cartoon Representing the Mammalian Pol II Holoenzyme

The core enzyme, consisting of 12 subunits, is associated with GTFs to form the transcription initiation competent holoenzyme. Only those GTFs are shown for which the presence of at least one subunit has been directly documented (Figure 1B). For multimeric GTFs, the examined subunits are indicated. The topological arrangement of GTFs within the complex are unknown, but direct interactions between TBP and TFIIB, CTD, and TAFs have been reported (see text).

for the immunoprecipitation. We would thus not be surprised if, in vivo, the majority of the above mentioned polypeptides were assembled into a large pol II holoenzyme complex, as schematically shown in Figure 7. It should be mentioned in this context that MO15/CDK7 may interact with pol II not solely via its association with TFIIH. In an immobilized protein interaction assay, the putative yeast homolog of MO15/CDK7, KIN28, binds directly to RPB1 and RPB2, the two largest pol II subunits (Feaver et al., 1994). Conceivably, the pol II holoenzyme complex may contain additional constituents, for example the mammalian counterparts of yeast SRB proteins (for review see Koleske and Young, 1995). This issue can be addressed as soon as antibodies for these constituents become available.

Since the antibody used in the immunoprecipitation was directed against MO15/CDK7 and since this kinase is tightly associated with TFIIH, one would expect higher recoveries for MO15/CDK7 and the TFIIH subunit p62 than for other components of the holoenzyme. Although this is indeed the case, it is well possible that MO15/CDK7 (and its associated subunits) also exists as separate entities that perform tasks unrelated to transcription. As mentioned above, the MO15/CDK7–cyclin H–p36 complex had originally been discovered as a CDK-activating kinase. Using in vitro assays, no MO15/CDK7 kinase activity changes could be monitored during cell cycle progression (Tassan et al., 1994). Hence, the precise role, if any, of this kinase in proliferation control is still unclear. But, MO15/CDK7, in its association with TFIIH, may have yet another function. Egly and coworkers (Roy et al., 1994) have proposed that this kinase may play a role in nucleotide excision repair, both in vivo and in vitro. It will be an enticing but difficult endeavor to determine whether, in vivo, different MO15/CDK7 complexes are involved in transcription and, perhaps, cell cycle regulation and DNA repair.

As revealed by kinase assays with GST–CTD fusion protein, MO15/CDK7 appears to phosphorylate the CTD in a cooperative manner. We thus suspect that the initial phosphorylations trigger the rapid and complete phosphorylation of the CTD. This begs the question of why the

initiation competent pol IIA isoform is unphosphorylated in spite of its close association with MO15/CDK7. The most straightforward explanation implies kinetic arguments. In rat liver, free unphosphorylated pol II (pol IIA) is far less abundant than transcriptionally engaged, phosphorylated pol II (pol IIO) (Wuarin and Schibler, 1994), suggesting a rapid turnover of the pol IIA pool. It is tempting to speculate that CTD dephosphorylation is closely associated with transcription termination. Released pol II, once assembled into a holoenzyme, may spend very little time before reinitiating transcription. This short transit time may not suffice for CTD phosphorylation.

Negative superhelical turns have previously been reported to facilitate a step in transcription initiation that, with linear templates, requires the activity of TFIIE, TFIIF, and TFIIH (Parvin and Sharp, 1993). Originally, this step was believed to be isomerization. However, by using an abortive transcription assay, Goodrich and Tjian (1994) have demonstrated that negative supercoils assist promoter escape, rather than isomerization. Although the rat liver holoenzyme favors circular superhelical templates somewhat over linear templates, it is clearly capable of initiating on both templates.

Two albumin promoters, one containing all of the upstream sequences for maximal in vitro transcription and one truncated at position −59, were similarly active in promoting transcription by the pol II holoenzyme. This is in accordance with the observation that transcriptional activators important for albumin in vitro transcription, such as HNF1 and C/EBPα, did not copurify with the holoenzyme. At least some of the holoenzyme molecules also contain TAF_{250}, TAF_{80}, and TAF_{55}. Given the high biochemical stability of the mammalian TFIID complex (Dynlacht et al., 1991), we suspect that additional TAFs are also coimmunoprecipitated with the MO-1.1 antibody. Since TAFs are important targets for transcriptional regulation, we considered that the holoenzyme may respond to transcriptional activators. However, our first attempts with supplementation of recombinant C/EBPβ were fruitless. While this protein efficiently stimulates transcription in unfractionated liver or spleen nuclear extracts (Descombes et al., 1990; V. O., unpublished data), it did not increase transcription by the holoenzyme. At present, several explanations can be offered for the failure of the holoenzyme to respond to E. coli derived C/EBPβ. For example, C/EBPβ may require posttranslational modifications or the aid of coactivators (such as CREB-binding protein, CBP; Kwok et al., 1994) that are not provided by the purified holoenzyme. Clearly, many additional experiments are required before we can reach firm conclusions with regard to these issues.

The existence of a pol II holoenzyme has first been established in yeast, through genetic and biochemical studies by the groups of R. Young and R. Kornberg (see Introduction). Unlike its yeast counterpart, the mammalian holoenzyme described in this paper does not require supplementation with TBP and TFIIE for successful in vitro transcription, as it already contains these components. We consider unlikely the possibility that this apparent difference is physiologically relevant. Rather, it may be the result of the different techniques used in the preparation of the two holoenzymes. The yeast pol II holoenzyme was enriched by extensive chromatographic fractionation of a whole-cell lysate (Koleske and Young, 1994; Kim et al., 1994), while the rat liver enzyme was obtained by a rapid immunopurification from concentrated extracts of highly purified nuclei. Perhaps, the interactions of pol II with TFIID and TFIIE are particularly susceptible to dilution and are broken during the lengthy purification procedure applied to the yeast enzyme. In this context, we should mention that our nuclei purification protocol involves the mechanical breaking of cells in a buffer containing 2 M sucrose (see Gorski et al., 1986). The high viscosity of this lysis buffer reduces diffusion, thereby minimizing leakage of nuclear components into the cytoplasm during subcellular fractionation. It is possible that the techniques commonly used for cells grown in tissue culture, such as HeLa cells, would not readily allow the isolation of a transcriptionally competent holoenzyme. In a HeLa whole-cell extract, the components of the basal transcription machinery may not be sufficiently concentrated to maintain the integrity of the holoenzyme, while nuclear extracts, obtained from cells lysed by hypotonic shock, may be depleted in some components of the basal transcription machinery.

Several observations point toward a crucial role of the CTD in the assembly of pol II holoenzyme. First, the mutant phenotype of yeast cells expressing RNA polymerase with truncated CTDs can be suppressed by mutations in SRB protein genes (Koleske et al., 1992; Thompson et al., 1993; Liao et al., 1995). Second, mammalian pol II with a truncated CTD responds poorly to most activators (Gerber et al., 1995). Third, only RPB1 subunits with intact (or nearly intact) CTDs were recovered in the immunoprecipitation with the MO15/CDK7 antibody.

Our work cannot exclude the possibility that binding of an integral holoenzyme to the promoter and stepwise assembly of several of its components at the promoter can occur in parallel. However, the frequently observed synergistic activation by multiple transcription factors could be more readily explained by involving a pol II holoenzyme. Such a large complex would offer more peptide surfaces interacting simultaneously with distinct transcriptional regulatory proteins and would thus be a better partner for cooperative binding than its individual components (for review see Ptashne, 1988). Moreover, the existence of a transcription competent eukaryotic pol II holoenzyme would unify the concepts of transcription in eukaryotes and prokaryotes, in spite of the enormous difference between the complexity of holoenzyme subunit structures.

Experimental Procedures

Nuclear Extract Preparation

Highly purified rat liver nuclei were prepared as described by Lichtsteiner et al. (1987). The nuclear pellet was resuspended at 10 OD_{260}/ml in nuclear lysis buffer containing 10 mM HEPES (pH 7.6), 100 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 0.1 mM NaF, 0.1 mM Na_3VO_4, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, and 10% glycerol). NaCl was added to a final concentration of 0.4 M to extract soluble nonhistone proteins. After 20 min on ice, the nuclear lysate was spun

at 36,000 rpm in a fixed angle Ti60 rotor for 60 min at 0°C to sediment insoluble chromatin components. Soluble proteins were precipitated by the addition of 0.3 g of solid $(NH_4)_2SO_4$ per ml of supernatant. After 1 hr on ice, the ammonium sulfate precipitate was recovered by sedimentation at 36,000 rpm in a fixed angle Ti60 rotor for 30 min at 0°C. The pelleted proteins were resuspended in dialysis buffer (25 mM HEPES [pH 7.6], 100 mM KCl, 0.1 mM NaF, 0.1 mM Na_3VO_4, 0.1 mM EDTA, 0.25 mM DTT, and 10% glycerol), in 5% of the original nuclear lysate volume, and dialyzed twice for 2 hr against 250 vol of the same buffer. After a 2 min centrifugation in an Eppendorf microfuge, the supernatant was divided into 100 μl aliquots, snap-frozen in liquid nitrogen, and kept under liquid nitrogen until use. The protein concentration was determined as described in Gorski et al. (1986). Typically, 1 g wet weight of rat liver yields 100 μl of nuclear extract containing 8–12 mg of protein/ml.

Holoenzyme Immunopurification

Per assay, 0.5 μl of ascites fluid containing a monoclonal anti-human MO15 antibody (MO-1.1, Tassan et al., 1994) or control antibody was incubated with 20 μl of a suspension containing magnetic beads coated with goat IgG anti-mouse IgG (Dynal M450) for 4 hr at 4°C. The beads were extensively washed in PBS containing 0.1% Triton X-100 and incubated with 50 μl of undiluted crude nuclear extracts for 1 hr at 4°C with gentle shaking. Subsequently, the beads were then washed three times with 400 μl of washing buffer (25 mM HEPES [pH 7.6], 50 mM KCl, 0.1 mM EDTA, 0.1 mM Na_3VO_4, 0.1 mM NaF, 10% glycerol, 0.1% Triton X-100).

In Vitro Transcription

In vitro transcription reactions were performed as described by Gorski et al. (1986) with either crude nuclear extract (20 μl assays) or purified holoenzyme immobilized on washed beads (10 μl to 20 μl assays). The assays contained typically 2 μg of template DNA and were incubated for 90 min at 37°C with gentle shaking.

Plasmid Constructions

The plasmid −59Alb400 was constructed from the plasmid −170 mut B Alb400 (Lichtsteiner and Schibler, 1989) by removing the sequences between −170 and −60. The plasmid −170 mut B Alb400 was digested with the restriction endonucleases EcoRI and BglII, the resulting 5′ overhangs were filled in with Klenow polymerase, and the resulting blunt-ended DNA was religated with T4 ligase, before being used to transform competent E. coli cells. The plasmid −170Alb220 was obtained by removing approximately 180 bp of the G-free cassette from the SmaI-digested plasmid −170Alb400 with ExoIII and mung bean nuclease (Zhu and Clark, 1994).

Protein Phosphorylation Assays

The GST–CTD fusion protein was expressed and purified from E. coli as described (Peterson et al., 1992; V. O. and U. S., unpublished data) and used as substrate (250 ng) in a reaction of 20 μl containing the MO-1.1 IP from 5 μl of nuclear extract, 20 mM HEPES (pH 7.6), 25 mM $MgCl_2$, 8 mM $CaCl_2$, 0.25 mM DTT, 10 nM ATP corresponding to 10 μCi [γ-^{32}P]ATP, with or without unlabeled ATP where indicated. The reaction was allowed to proceed for 15 min at 37°C, stopped by the addition of 2× Laemmli buffer, and the proteins were separated by SDS–PAGE (Laemmli, 1970).

Other Methods

Western blot analysis was done as described in Ossipow et al. (1993), except that immunocomplexes were visualized by using goat anti-mouse or goat anti-rabbit antibodies coupled to horseradish peroxidase by using the Amersham ECL kit. Antibodies against TAF_{250}, TFIIEα, and TFIIB were purchased from Santa Cruz Biotechnology and used at a dilution of 400-fold. In the Western blot experiments with the other antibodies (provided by colleagues, see Acknowledgements), the following dilutions were used: 10,000-fold for TBP, MO-1.1, and p62; 5,000-fold for CTD; 2,000-fold for TAF_{80}, TAF_{55}, $TFIIF_{RAP30}$, HNF1, HNF4, and C/EBPα. Templates for in vitro transcription were prepared as described by Gorski et al. (1986). Recombinant human TBP was prepared as described by Chiang et al. (1984) and was used in electrophoretic mobility shift assays with a double-stranded oligonucleotide (upper strand, 5′-CCTGAAGGGGGGCTATAAAAGGGGGT-3′) as described (Ossipow et al. 1993).

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