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ISOLATION AND IDENTIFICATION OF HUMAN RNA POLYMERASE II-ASSOCIATING PROTEINS

By

Shu-Meng Maurice Lin

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ABSTRACT

ISOLATION AND IDENTIFICATION OF HUMAN RNA POLYMERASE II-ASSOCIATING PROTEINS

By

Shu-Meng Maurice Lin

Calf thymus RNA polymerase II was covalently immobilized on agarose and used to affinity purify human RNA polymerase II-associating proteins (RAPs). This technology was previously used to isolate human RAP30/74 (TFIIF) and RAP38 (TFIIS, SII). RAP30/74 is an initiation and elongation factor for transcription by RNA polymerase II, and RAP38 is an elongation factor. Using antibodies directed against subunits of the initiation factor TFIIE, this factor has now been identified as a RAP (RAP34/56). Initiation factor TFIIB was not detected in RAP fractions by Western blot analysis. By comparison of RAP and control fractions, several novel RAPs have been tentatively identified and designated RAP110, 87, 69, 50, 32, and 25 (the number indicates apparent molecular weight in kilodaltons). These RAPs have been isolated to near homogeneity for microsequencing. A tryptic peptide derived from RAP50 has been sequenced. Searching of nucleic acid and protein sequence databases shows that this peptide matches a fragment of human translation elongation factor-1α (EF-1α) (53-kDa).

DEDICATION

To the Lord Jesus, and my parents.

ACKNOWLEDGMENTS

I would like to thank Dr. Zachary F. Burton from Department of Biochemistry for his guidance and criticism. I would like to thank Dr. Janek Werel and Richard Fentzke for their contribution to my project. I also wish to thank Dr. Davis, Dr. Gerlach, and Dr. Thorne for their committee guidance. This work is done under the support of NIH and Michigan State University Department of Biochemistry.

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Chapter I. Literature Review

Differentiation and development are largely controlled at the level of transcription. Therefore, it is very important to understand the mechanism and regulation of transcription. RNA polymerases are the enzymes that catalyze the transcription process. There are three distinct RNA polymerases in eukaryotic cells. RNA polymerase I, II, and III transcribe genes encoding rRNA, mRNA, and tRNA, respectively. All these RNA polymerases require protein transcription factors (TF) to initiate transcription. For example, RNA polymerase III requires at least three factors, TFIIIA, TFIIIB, and TFIIIC (1). There are also factors that regulate promoter escape, elongation, termination, capping, and splicing. This work focuses on the factors in the human RNA polymerase II system.

Transcription by RNA Polymerase II

RNA polymerase II transcription is a complex, multistep process that can be divided into three stages : initiation, elongation and termination. Initiation encompasses the location of a promoter by RNA polymerase II, the formation of the first phosphodiester bond, and the escape from the promoter. During the elongation stage, RNA polymerase II catalyzes the processive addition of ribonucleotides to the 3' end of the growing RNA chain until specific attenuation or termination signals are encountered. Finally, transcription is terminated and polymerase is released from the DNA template. An understanding of transcription initiation by RNA polymerase II has been facilitated by a combination of *in vivo* and *in vitro* studies. However, the elongation and termination stages of transcription are not as well understood.

The initiation stage is a major site for regulation. In eukaryotes, initiation is regulated by several functional classes of DNA sequence elements such as core promoter elements, upstream promoter elements, and enhancers. Core promoter elements contain the binding site for RNA polymerase II, and control the location of the initiation site. Upstream promoter elements and enhancers regulate the initiation rate from the core promoter. Two classes of transcription factors are involved with those DNA sequence elements : general initiation factors, also named basal initiation factors, and regulatory factors. General initiation factors are essential for initiation and are sufficient to initiate a basal level of transcription from many core promoters. Regulatory factors bind to upstream promoter elements and enhancers, and modulate the rate of transcription initiation (2-9).

Unlike bacterial RNA polymerases which are purified as holoenzymes tightly associated with their initiation factors (10-14), purified RNA polymerase II is separated from its initiation factors during purification procedures. Therefore, identification, purification, and functional analysis of initiation factors are crucial in studying the initiation and regulation of eukaryotic mRNA synthesis. Roeder and colleagues first demonstrated that initiation by RNA polymerase II requires multiple initiation factors (15, 16). These findings have been confirmed and extended by many labs in several organism systems including the human system (17-19). Many general initiation factors have been purified, their genes have been cloned, and their roles have been defined. For example, TFIID, IIA, IIB, IIF, IIE, IIH, and IIJ have been identified. These general initiation factors appear to be required for all core promoters, whether or not the promoter contains a TATA box.

The Structure of Class II Promoters

Accurate transcription by RNA polymerase II is entirely dependent on the presence of promoter-containing eukaryotic DNA as signals that direct transcription factors and RNA polymerase II to the initiation site (15, 17). Three classes of cis elements have been identified in the promoters of class II genes : the TATA box, the initiator (Inr) region, and upstream elements. The TATA box and the Inr region are core promoter elements. One or both of them are present in all protein-coding genes, and each is independently capable of transcription complex formation. Upstream elements are variable elements whose presence and absence are gene specific.

RNA Polymerase II

RNA polymerase II ,which transcribes mRNA and small nuclear RNA (snRNA), has been purified from many sources (20, 21). It is a 500 to 600-kDa multisubunit enzyme. Calf thymus RNA polymerase II contains 12 subunits with molecular weights ranging from 210 to 11.5-kDa (22). Hela cell RNA polymerase II contains 10 subunits with molecular weights ranging from 240 to 10-kDa (20, 21). The largest subunit, which is homologous to the ß' subunit of *E.coli* RNA polymerase (23), contains an unusual C-terminal domain (CTD) with 52 repeats of the consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser in mouse (24) and hamster (25). Other eukaryotic species have various numbers of CTD repeats. For example, it is repeated 26 times in yeast (23) and 45 times in Drosophila (25). The CTD is not present in prokaryotic RNA polymerase or in RNA polymerase I or RNA polymerase III. This domain can be multiply phosphorylated on the first and second serine residues of the consensus sequence by kinases (26). As a result of this phosphorylation, the largest subunit can be resolved as two forms on gels. The II_a form is the unphosphorylated form with an apparent molecular weight of 215-kDa (27). The form II_0 is the phosphorylated form with an apparent molecular weight of 240-kDa (27). Another form, II_b, is 180-kDa (27) and is a proteolyzed form of the largest subunit from which the CTD has been removed (23, 24). The second largest subunit II_c is homologous to the ß subunit of *E. coli* RNA polymerase (28).

CTD - Since deletion mutants that lost more than half of the repeats in CTD are lethal in mouse, Drosophila, and yeast, the CTD has an essential role *in vivo* (25, 29-31). It has been demonstrated that the CTD specifically interacts with TFIID (32), and there is evidence showing that these repeats can interact with the activation domains of some transcription regulatory proteins (33). Therefore, it is believed that the function of the CTD is to mediate transcription activation by upstream regulators. One report indicates that the CTD can intercalate into DNA nonspecifically (34). This suggests that the CTD may strengthen RNA polymerase II binding and initiation complex stability.

The other functional significance of the CTD is mediated by phosphorylation. Dahmus and Laybourn (35) found (a) the unphosphorylated form, II_a, stably associates with the preinitiation complex, (b) the phosphorylated form, II_0, can be isolated from active elongation complexes, and (c) the conversion of form II_a to II_0 occurs prior to formation of the first phosphodiester bond. It suggests that the unphosphorylated polymerase preferentially assembles into the preinitiation complex, and subsequent phosphorylation of the CTD is important for the transition from initiation to elongation. Phosphorylation of the CTD in the initiation complex decreases the affinity of RNA polymerase II for TATA binding protein (TBP) (35) and, thus, may release polymerase facilitating promoter escape (36).

CTD Kinases - Dahmus and Laybourn (35) proposed that the CTD kinase may be one of the general initiation factors. TFIIH was found to contain a specific CTD kinase activity stimulated by DNA elements that can direct the formation of a transcription complex (37). Dynan and colleagues have isolated a kinase specific for the CTD that depends on nonspecific DNA for phosphorylation (38).

Assembly of the Preinitiation Complex

Eukaryotic RNA polymerase II is responsible for mRNA transcription and it requires basal transcription factors to initiate transcription accurately from a promoter. In the case of the adenovirus major late promoter, which contains a TATA box and Inr., it requires at least seven factors : TFIID, TFIIA, TFIIB, TFIIF, TFIIE, TFIIH, and TFIIJ (Table 1). Transcription initiation is preceded by the orderly association of DNA template, RNA polymerase II, and these factors (Figure 1). TATA binding protein (TBP), which is a subunit of the TFIID complex, recognizes the TATA sequence and binds first to the promoter to form a TFIID-DNA complex (39). Then TFIIA binds TFIID forming a DA complex (abbreviated DA complex). Subsequently, TFIIB associates with the DA-DNA complex to form a DAB complex (40, 41). In next step, RNA polymerase II is delivered by TFIIF resulting in the formation of DABPolF complex. RNA polymerase II must bind TFIIF to enter the complex (42). TFIIE followed by TFIIH binds DABPolF to form the DABPolFEH complex. Finally, TFIIJ binds, to complete the transcription preinitiation complex (Figure 2). This complex can be converted into an elongation complex after ATP hydrolysis, DNA strand separation, first phosphodiester bond formation, and promoter escape.

Transcription Initiation Factors

TFIID - Native TFIID from higher eukaryotes is a high-molecular-mass, multisubunit complex composed of TBP and additional polypeptides named TATA-associating factors (TAFs) (43, 44). Although native TFIID has been reported to be 120 to 140-kDa (45, 46), it is now believed to be larger than 700-kDa (18, 48-50).

Cloned TBP is a 38-kDa protein composed of a highly conserved C-terminal domain (43, 44). This domain contains two direct repeats connected by a lysine-rich spacer region. The direct repeats are similar in sequence to bacterial σ factor region 2.4

Factor	Peptide composition	Cloned gene	in RAP fraction
IID	38kD of TBP and TBP associating factors (TAFs)	yes (TBP only)	no TBP
IIA	34kD, 19kD, and 14kD	yes	not identified
IIB	35kD	yes	no*
IIF	30kD dimer and 74kD dimer	yes	RAP30/74
IIE	34kD and 56kD	yes	RAP32/55*
IIH	90kD, 62kD, 43kD, 40kD, and 35kD	90 and 62 kD	not identified
IIJ	?	no	not identified

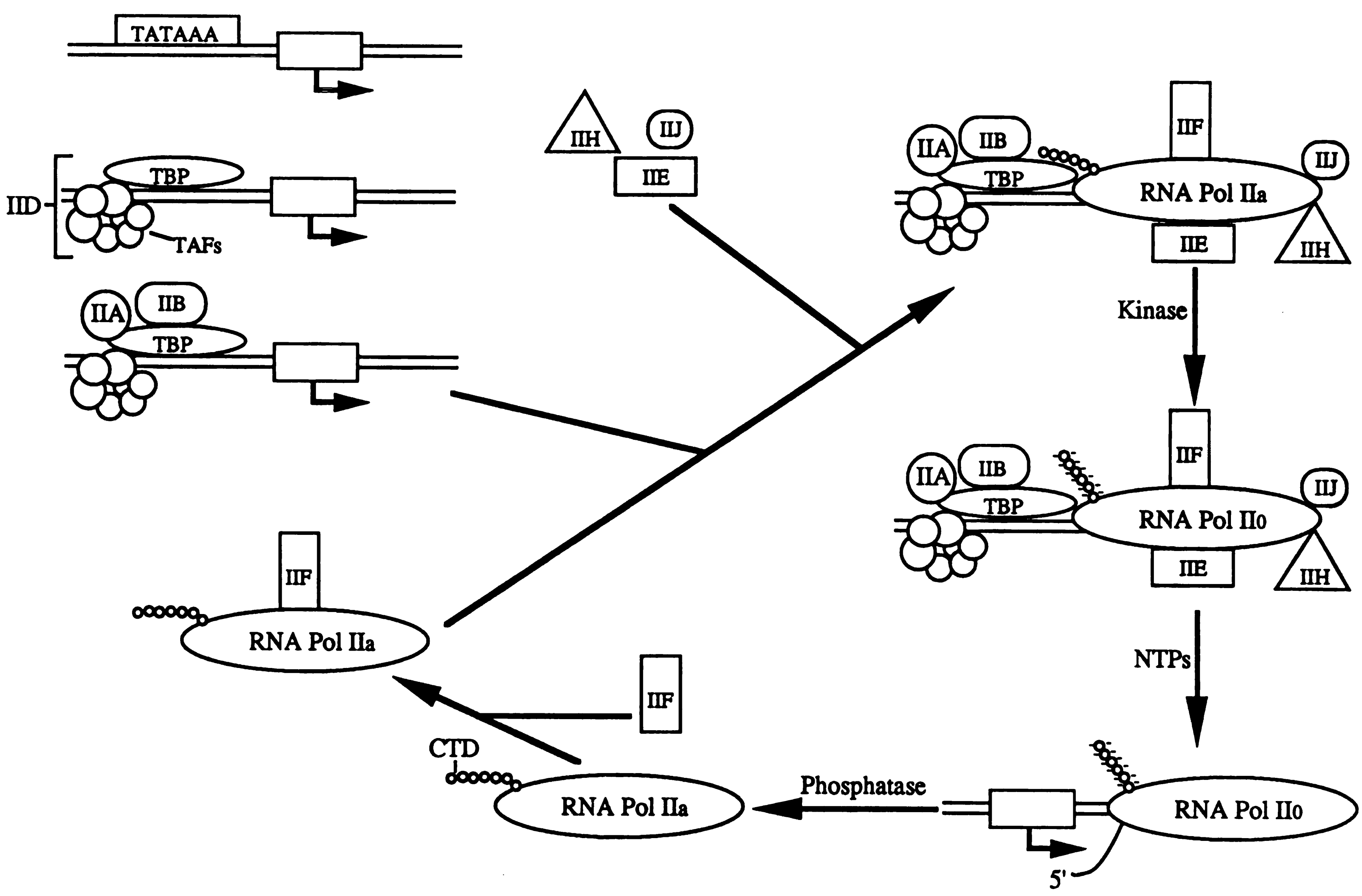
* This thesis

Table 1. General initiation factors isolated from Hela cell extracts.

Figure 1. Formation of the preinitiation complex on a TATA-containing promoter.

The model depicts the order of assembly of the general initiation factors and the transition from initiation to elongation as mediated by a CTD kinase. This model indicates that the unphosphorylated form II_a of RNA polymerase II associates with the preinitiation complex. The action of a CTD kinase that converts II_a form to II_0 form RNA polymerase II is thought to be at least one event to activate the complex. Transcription begins when NTPs are supplied to an activated complex. The action of a phosphatase that converts II_0 form to II_a form RNA polymerase II is required for RNA polymerase II to re-enter the cycle after termination.

Fig. 1


TATAAA
IID
TBP
TAFs
IIA
IIB
TBP
IIH
IIJ
IIE
IIF
RNA Pol IIa
IIF
CTD
RNA Pol IIa
IIA
IIB
TBP
IIF
RNA Pol IIa
IIJ
IIE
IIH
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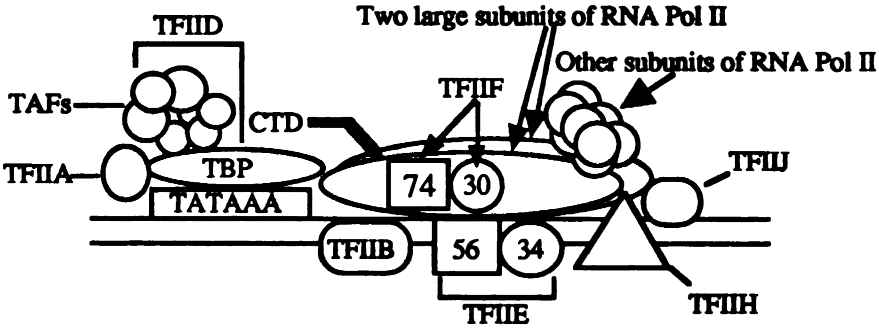


Figure 2. Preinitiation Complex on a TATA Promoter.

(51-53) which interacts with the -10 element of bacterial promoters, TATAAT, which is similar to the sequence TATAAA found in eukaryotic promoters, which is bound by TBP (14, 54). Therefore, the conserved C-terminal domain appears to be the DNA-binding region. TBP has two DNA-binding properties. First, association and dissociation between TBP and the TATA box are very slow (55, 56). Second, TBP binds as a monomer (57) to the minor groove of promoters (58, 59). Since specific interactions have been shown between the acidic activator VP16 and TFIID (60, 61), TFIID is likely to be one of the targets of transcriptional regulators.

At least seven human TAFs of 250, 150, 120, 100, 70, 40, and 30-kDa have been identified by immuno-affinity purification procedures using anti-TBP antibodies (62). TAFs which associate tightly with TBP have been proposed to participate in promoter recognition and to mediate interaction of TFIID with transcriptional regulatory factors (43, 44).

TFIIA - Due to different procedures, human TFIIA has been purified from Hela cells as a 43-kDa protein (63), a 38-kDa protein (64), or a trimer composed of 34, 19, and 14-kDa subunits (65). Surprisingly, the 43-kDa protein has similar properties with actin including the molecular weight and the antibody cross-reaction (63). TFIIA promotes stable binding of TFIID to the core promoter (19, 45, 66, 67). Whether TFIIA is an essential initiation factor is unclear. TFIIA appears to be required for transcription in crude or regulated systems but not in more highly purified systems (48, 68). TFIIA is thought to be an anti-inhibitor with an antagonistic function with repressors such as DR-1 and DR-2 (65), which are present in extracts.

TFIIB - TFIIB purified from human cells contains a single 35-kDa polypeptide (69-71). A cDNA encoding human TFIIB has been isolated (69, 70). TFIIB does not bind DNA or have any known enzymatic activity (68, 72). However, it has been shown to be required for selective binding of RNA polymerase II to the TFIID-DNA complex (67, 73, 74). Other findings are : (a) gel shift assays show that TFIIB is able to interact with TFIID-DNA complexes (67, 71, 75, 76); (b) TFIIB associates stably with RNA polymerase II *in vitro* (77). These characteristics indicate that TFIIB may play a role in "measuring" the distance from TATA box to the transcriptional start site (67, 77, 78). Specific interactions have been shown between the acidic activator VP16 and TFIIB (79, 80) suggesting that TFIIB is likely to be another target of transcriptional regulators.

TFIIF - This factor also known as RAP30/74 is composed of 30 and 74-kDa (58-kDa by sequence) subunits designated RAP30 and RAP74, respectively. These subunits, which have been purified from human cells (81, 82), were first identified by Greenblatt and colleagues fractionating calf thymus or Hela cell extracts over columns containing immobilized RNA polymerase II (83-86). cDNAs encoding both RAP30 (87-90) and RAP74 (91, 92) have been isolated. Transcriptional activity of TFIIF elutes from a gel filtration column with an apparent molecular mass of 220-kDa, suggesting that RAP30/74 is a heterotetramer (93).

Both subunits are required for transcriptional activity (81, 82, 91, 92, 94). Based on the observation that recombinant RAP30 binds immobilized RNA polymerase II in the absence of RAP74. TFIIF appears to bind RNA polymerase II only through the RAP30 subunit (95). Other evidence shows that interaction of RAP30 with RNA polymerase II is stabilized by RAP74 (90).

TFIIF has been shown to act with TFIIB to promote selective binding of RNA polymerase II to the TFIID-DNA complex (73, 74, 96, 97). The observation that TFIIF binds to *E. coli* RNA polymerase and can be displaced by σ^{70} suggests that TFIIF and σ^{70} may have related RNA polymerase-binding domains (98). Analysis of the amino acid sequence of RAP30 reveals a region of sequence similarity with RNA polymerase binding domains of *E. coli* σ^{70} and *Bacillus subtilis* σ^{43} (87, 98). This region may physically contact polymerase.

TFIIF has been reported to prevent nonselective binding of RNA polymerase II to free DNA (95, 99), much as *E. coli* σ^{70} prevents nonselective binding of *E. coli* RNA polymerase to nonpromoter sites on DNA. How TFIIF controls binding of RNA polymerase II to DNA is not clear. Suppression of nonspecific binding may be a property of RAP30, since it can be achieved with recombinant RAP30 (95). Because RAP30 itself is not able to dissociate pre-formed binary complexes of RNA polymerase II at nonspecific sites, it is likely that both RAP30 and RAP74 play an important role in this process (95). In addition to its requirement for transcription initiation, TFIIF has the ability to stimulate transcription elongation (93). The RAP74 subunit of TFIIF has been shown to be required for the escape of RNA polymerase II from the promoter (100).

TFIIE - This factor, which has been purified from Hela cells (101, 102), is composed of 34 and 56-kDa subunits. cDNA clones encoding both subunits have been isolated

(103, 104). On the basis of its apparent molecular mass of 200-kDa determined by gel filtration (101, 102), human TFIIE is thought to be a heterotetramer. Both subunits have been shown to be required for transcriptional activity (49, 103-105). TFIIE can associate stably with the preinitiation complex after the association of RNA polymerase II and TFIIF (65, 67, 102, 103, 106, 107). Since TFIIE has been reported to associate stably with RNA polymerase II in solution (97, 108), the assembly of TFIIE into the complex may be mediated, in part, through an interaction with RNA polymerase II.

TFIIH - This factor was first purified from rat liver with polypeptides of 94, 85, 68, 46, 43, 40, 38, and 35-kDa (106, 109), and subsequently purified from human cells with polypeptides of 90, 62, 43, 40, and 35-kDa (110). Monoclonal antibodies specific against the 62-kDa subunit of human TFIIH cross-react with the 68-kDa subunit of rat TFIIH. Human TFIIH is a multi-subunit protein with an estimated molecular mass of 200-kDa determined by glycerol gradient sedimentation (110). cDNAs encoding the 62-kDa (111) and 90-kDa (114) subunits of human TFIIH have been isolated. It has been demonstrated that TFIIH is able to assemble into the preinitiation complex (106, 107, 112), and to associate stably with RNA polymerase II in solution (110).

TFIIH has been shown to have a DNA-dependent ATPase activity (109). This ATPase has the following properties : (a) it has low specific activity; (b) it can be preferentially stimulated by DNA fragments containing promoter sequences; and (c) it requires ATP. In addition to ATPase activity, TFIIH has a CTD kinase activity which is able to phosphorylate the C-terminal domain of the largest subunit of RNA polymerase II (37, 113). This activity is strongly stimulated by the presence of other initiation factors and promoter DNA (37). More resently TFIIH has been reported to have an ATP-dependent helicase activity (114). The 90-kDa subunit of TFIIH is encoded by the human ERCC-3 gene (114), which is mutated in individuals suffering from xeroderma pigmentosum and Cockayne's syndrome. Apparently this general transcription factor has a role in ultraviolet DNA mutation repair.

TFIIJ - This factor is separated from TFIIA during hydroxylapatite chromatography when Cortes and colleagues were extensively purifing TFIIA from Hela cells (65). The peptide composition of TFIIJ has not been reported. TFIIJ is required for transcription when bacterially produced TBP is used, but only has a modest effect with native TFIID (65). It appears to be the last factor to assemble into the preinitiation complex (65).

Transcription Elongation by RNA Polymerase II

A number of factors that influence elongation and termination by prokaryotic RNA polymerase have been defined (115). In particular, the N and Q protein-mediated antitermination systems of λ and related bacteriophages provide a model for how specific gene expression can be controlled by modifying RNA polymerase elongation. A key feature in this process is the pausing of the RNA polymerase downstream of the initiation site where Q protein is added to the elongation complex with the aid of another elongation factor NusA (116, 117). This Q-modified RNA polymerase is then able to read through downstream pause sites and termination sites of both the ρ-dependent and ρ-independent varieties.

Unlike the initiation stage in eukaryotes and the elongation stage in prokaryotes, the elongation stage of transcription is not well understood. However, it is clear that the transcription of eukaryotic genes is controlled during the elongation stage as well. This control mechanism has been shown to occur by attenuation of transcription within a number of cellular genes such as *c-myc*, *c-myb*, *c-fos*, and adenosine deaminase (118). Human Immunodeficiency Virus Type I (HIV-1) encodes a transcriptional regulatory protein, Tat, which regulates HIV-1 gene expression by increasing transcription initiation and by increasing the efficiency of elongation (119-124). A possible mechanism for elongation regulation by Tat is that Tat engages RNA polymerase via a complex formed with at least one cellular factor and a stem loop structure (tar) adopted by the nascent viral RNA transcript (119).

Several groups have explored the factor requirements for efficient transcription elongation by RNA polymerase II *in vitro* (45, 93, 125-134). At least four protein factors that affect the elongation characteritics of RNA polymerase II have been identified in higher eukaryotes. SII (37-kDa), also named TFIIS and RAP38, has been found to suppress pausing by RNA polymerase II at specific sites (127, 128, 132, 135, 136). TFIIF, which is also a basal initiation factor, as described above, stimulates the elongation rate of RNA polymerase II (84, 93, 108, 129, 130). TFIIX which was identified in Hela cell extract stimulates elongation by RNA polymerase II (45, 137). P-TEF (positive transcription elongation factor), which was identified in *Drosophila*, is able to convert abortive elongation complexes to productive elongation complexes (138). Two of these elongation factors, SII (RAP38) and TFIIF (RAP30/74), have been identified as RNA polymerase II-associating proteins (86).

Pre-mRNA Processing

Capping - Presence of the 5'-terminal cap structure, $m^7G(5')pppN$, is a ubiquitous feature of eukaryotic mRNAs. This structure is required for initiation of protein synthesis and for stabilization of mRNAs (148, 149). An essential role of the cap structure in the mRNA splicing reaction has also been indicated (150-152). Capping is an early transcriptional event, occurring shortly after initiation (153-156). The enzyme which catalyses the capping reactions is mRNA capping enzyme (GTP : mRNA guanylyltransferase). This enzyme has been partially purified from rat liver (157, 158) Hela cells (159-161), and yeast (162, 163). The yeast capping enzyme, which has an approximate molecular weight of 180-kDa, is consisted of two subunits of 52 and 80-kDa possessing mRNA guanylyltransferase and RNA 5'-triphosphatase activities, respectively (164). The gene encoding the 52-kDa subunit has been isolated (165).

Polyadenylation - The 3'-ends of almost all eukaryotic mRNAs carry polyadenylate tails, which play an as yet undefined role in translation (166, 167). The regulation of poly(A) tail length is an important mechanism of translational control (168). The tail is formed by the addition of 200-250 adenylate residues to a 3'-end generated by endonucleolytic cleavage of the precursor RNA (169). Both cleavage and polyadenylation depend on the highly conserved sequence AAUAAA located 10-30 nucleotides upstream of the poly(A) addition site (169).

AAUAAA-dependent polyadenylation of pre-cleaved RNA can be reconstituted *in vitro* with only two factors : poly(A) polymerase, which catalyses the polymerization of the poly(A) tail, and cleavage and polyadenylation specificity factor (CPSF), which interact with the AAUAAA motif. Poly(A) polymerase, initially purified as a 60-kDa protein from calf thymus (170), was later shown by cDNA cloning to have a molecular weight of 82-kDa (171). Purified CPSF from calf thymus and Hela cells consists of four polypeptides with molecular weights of 160, 100, 73, and 30-kDa (172).

Splicing - Most protein-coding genes of higher eukaryotes contain intervening sequences, introns, that are precisely excised from their transcripts by a nuclear process known as pre-mRNA splicing. The first observation step in the *in vitro* splicing reaction is a cut at the 5' splice junction accompanied by formation of a 2'-5' phosphodiester bond between the 5' terminal G residue (5' splice site) of the intron and an A residue (branch site) located about 30 nucleotides upstream of the 3' end of the intron (3' splice site), forming a lariat structure (173-176). In the second step, splicing is completed by cleavage at the 3' splice site followed by ligation of the 5' and 3' exons to produce a spliced RNA and an excised intron still in the form of a lariat.

The splicing of pre-mRNA involves in the formation of a multicomponent complex, the spliceosome (177). This splicing body contains several small nuclear ribonucleoprotein particles (snRNPs) U1, U2, U4, U5, and U6. U1 snRNP contains U1 snRNA whose 5' end is complementary the 5' splice site region consensus sequence present in pre-mRNA introns (178, 179). U2 snRNP binds to sequences upstream of the 3' splice site that encompass the branch site (180, 181). The nature of the specificity for the binding of U4, U5, and U6 during the formation of the spliceosome structure is not clear.

In addition to snRNPs, several splicing factors have been identified in mammalian cells including U2AF (182), SF1, SF3 (183), ASF/SF2 (184, 185), and Sc35 (186). Only limited information is available about the exact function of these proteins. U2AF binds to sequence at the 3' end of an intron (187-189). ASF/SF2 is associated with general RNA-binding and RNA-annealing activities (185). Both U2AF and Sc35 are required for U2 snRNP to bind to the branch site (182, 190), and Sc35, in addition, plays a role in the initial of U1 snRNP to the pre-mRNA (190). Genes encoding U2AF (191) and Sc35 (192) have been isolated.

Chapter II.

INTRODUCTION :

RNA Polymerase II-Associating Proteins

The study of transcription in prokaryotes provides important models for understanding transcription in eukaryotic systems. Several proteins that bind to RNA polymerase II have been well understood to regulate transcription in bacteria. σ factors bind to RNA polymerase, and enable it to selectively initiate transcription at various kinds of promoters (14). In the case of *E. coli*, most promoters are recognized by σ^{70}. Following initiation of transcription, σ^{70} spontaneously dissociates from RNA polymerase (139) and the elongating RNA polymerase binds to another *E. coli* protein NusA (140). NusA serves several roles. It enhances pausing by RNA polymerase at certain sites and is important for termination at other sites. NusA also serves to couple certain bacteriophage antitermination factors, such as N and Q, to RNA polymerase (141). When transcription is terminated, RNA polymerase holoenzyme containing σ^{70} is reconstituted. Therefore, the bacterial transcription cycle is modulated by proteins that cyclically associate with RNA polymerase, and is highly regulated within this process.

Three RNA polymerase II-associating proteins (RAPs) have been demonstrated to have important roles in the eukaryotic transcription machinery. They are RAP30, RAP74, and RAP38, designated by their molecular weights in kDa (86). RAP30 and RAP74, which are identical to subunits of TFIIF, are required for initiation and have functions in elongation, as described in previous sections. RAP38 which is identical to elongation factor SII is able to stimulate elongation (127).

RNA Polymerase II Affinity Chromatography

To construct a RNA polymerase II affinity column, purified calf thymus RNA polymerase II was covalently immobilized to a gel matrix (Figure 3), whereas the control column has no attached protein ligand (86). Cell extracts were passed first through the control column, and then through the RNA polymerase II column. The proteins that bind

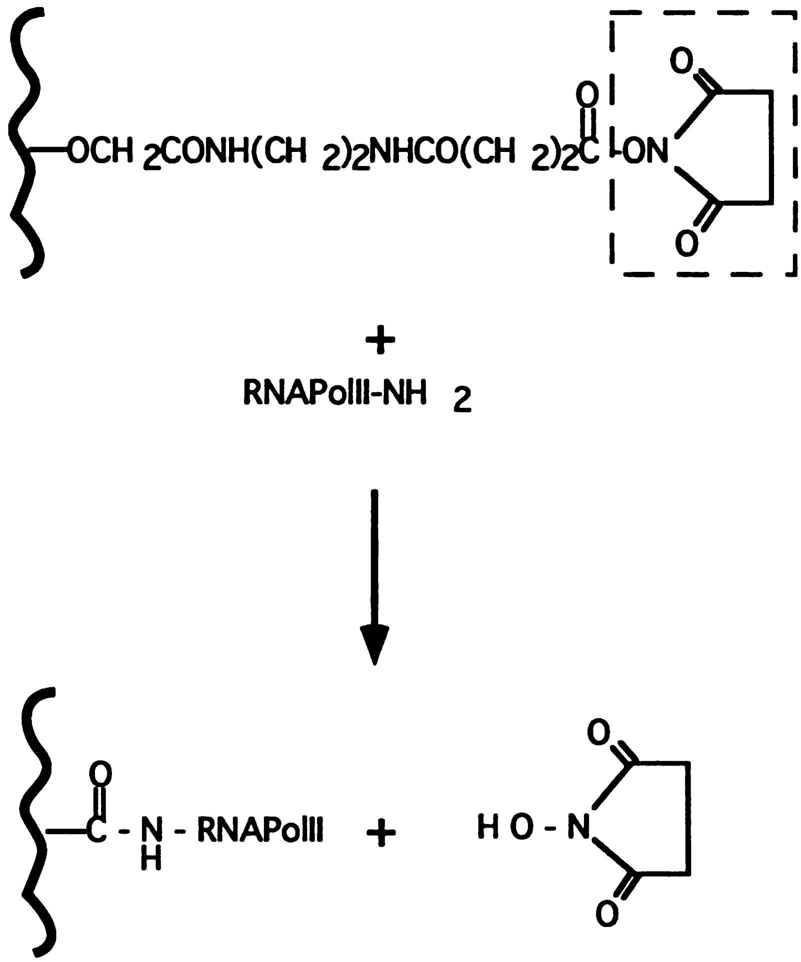


Figure 3. RNA Polymerase II Immobilization
(Coupling reaction of Affi-Gel 10 with ligand containing free amino groups)

specifically to the RNA polymerase II column are called RAPs for RNA polymerase II-associating proteins.

RNA polymerase II requires TBP, TFIIB, TFIIE, TFIIF, TFIIH, and TFIIJ, to initiate accurate transcription. In addition, more protein factors appear to be required for elongation and termination. However, Buratowski and colleagues showed that TBP, TFIIB, RNA polymerase II, and RAP fractions can reconstitute accurate transcription *in vitro* (97). Therefore, TFIIF, TFIIE, TFIIH, and TFIIJ are expected to be in the RAP fraction. In RAP fractions, our lab has previously identified 34-kDa, 35-kDa, and 55-kDa proteins whose molecular weights are similar to subunits of TFIIB and TFIIE purified from Hela cells by ion exchange chromatography. Protein factors related to transcription regulation, capping, polyadenylation, and splicing are expected to be associated with RNA polymerase II as well. In this work, RAP fractions were probed with anti-TFIIB, anti-TFIIE large subunit, and anti-TFIIE small subunit antibodies on Western blots. TFIIB was not identified in RAP fractions, but both TFIIE subunits are RAPs. Several RAPs were identified which did not correspond to any known protein.

METHODS :

The overall scheme of this work is shown in Figure 4.

Preparation of RNA Polymerase II

RNA polymerase II was purified from calf thymus, as described by Hodo and Blatti (22). One Kg of frozen calf thymus was thawed in 2 L of buffer A (10 mM Tris pH 7.9, 25 mM KCl, 50 mM EDTA, 10 mM EGTA, 12.5 % glycerol, and 0.5 % ß-mercaptoethanol) and homogenized in the Waring Blender for 30 sec twice at low, medium, and high speeds. Two L of buffer B (50 mM Tris pH 7.9, 50 mM EDTA, and 10 mM EGTA) was then added and blended for 30 sec each at low, medium, and high speeds. The homogenate was centrifuged at 8,000 rpm for 20 min in a Sorvall GS-3 rotor. To the supernatant, 4 µL per mL 10 % polyethylenimine was added to precipitate nucleic acids and proteins. After stirring on ice for 30 min, the solution was centrifuged, as described above.

The pellet, which contains nucleic acids and some proteins, was blended with 1 L buffer C (50 mM Tris pH 7.9, 10 % glycerol, 0.1 mM EDTA, 0.2 M ammonium sulfate, and 0.5 mM dithiothreitol). Additional ammonium sulfate was added to compensate for the pellet volume. This mixture was stirred on ice for 1 hr followed by centrifuging at 10,000 rpm for 20 min. Nucleic acids were removed by this step. Then 0.24 g per mL ammonium sulfate was added to the supernatant and stirred on ice for 1 hr. This is a protein precipitation step. The precipitate was collected by centrifuging at 30 K rpm for 30 min in a Beckman Ti-45 rotor.

The pellet was resuspended in buffer D (50 mM Tris pH 7.9, 25 % glycerol, 0.1 mM EDTA, and 0.5 mM dithiothreitol) and dialyzed (VWR dialysis tube of 12-kDa to 14-kDa cutoff) against the same buffer to a salt concentration below 150 mM ammonium sulfate. The dialysate was mixed in batch with 300 mL of DEAE-cellulose anion exchanger (DE-52, Whatman) and then washed 3 times with buffer D containing 150 mM ammonium sulfate. The washed resin was loaded into a 5x18 cm column. The column was washed with buffer D-150 mM ammonium sulfate until a stable background of absorbance at 280 nm was achieved. Protein was eluted with buffer D-500 mM ammonium sulfate. Bovine serum albumin was added to the protein peak to final

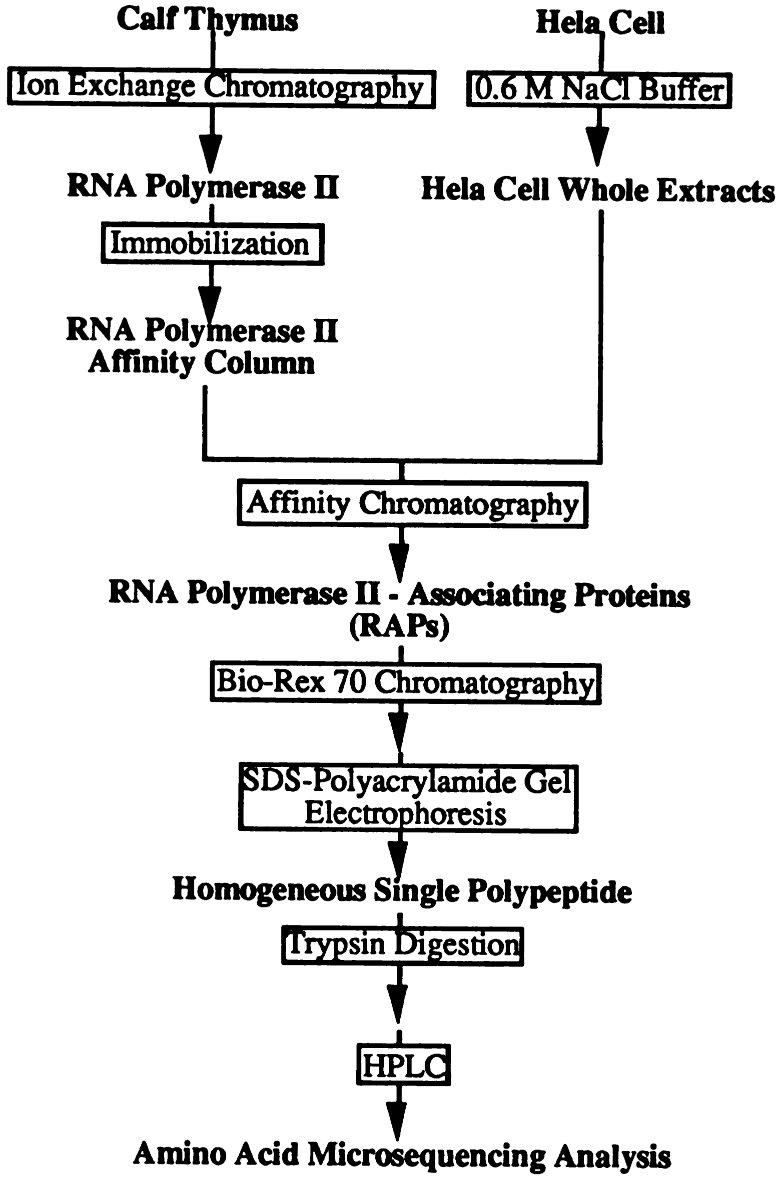


Figure 4. Overall Scheme.

concentration of 0.2 mg/mL followed by dialysis against buffer D to 50 mM ammonium sulfate.

The dialysate was loaded into a 60 mL bed volume (2.5x12 cm) of Phosphocellulose cation exchanger (P-11, Whatman) previously equilibrated with buffer D-50 mM ammonium sulfate with 0.2 mg/mL bovine serum albumin. The column was then washed with buffer D-50 mM ammonium sulfate. RNA polymerase II was eluted with buffer D-200 mM ammonium sulfate.

After dilution with Buffer D to 150 mM ammonium sulfate concentration, the preparation was loaded into a 5 mL bed volume DE-52 column. The column was washed with buffer containing 20 mM Hepes pH 7.9, 25 % glycerol, 0.1 mM EDTA, 100 mM NaCl, and 0.5 mM dithiothreitol. Enzyme was eluted with the same buffer containing 600 mM NaCl. This concentrated RNA polymerase II was dialyzed against storage buffer (20 mM Hepes pH 7.9, 0.1 M NaCl, 50 % glycerol, 0.1 mM EDTA, and 0.5 mM dithiothreitol) and stored in aliquots at -85° C.

Isolation of Form II_a RNA Polymerase II

Form II_a RNA polymerase II was purified by immuno-affinity chromatography (142). A part of primarily purified enzyme was mixed with 3 mL 8GW16 mAb/Sepharose (gift of Dr. N. Thompson and R. Burgess, McArdle laboratory for cancer research, U. of Wisconsin, Madison), which is CNBr activated Sepharose conjugated with monoclonal antibody against the RNA polymerase II CTD. After incubation with gentle inversion at 4°C for 1-2 hr, the mixture was transferred to a small column followed by washing with buffer containing 50 mM Tris pH 7.9, 0.1 mM EDTA, and 50 mM ammonium sulfate and the same buffer with 200 mM ammonium sulfate. The Form II_a RNA polymerase II was eluted with 30 % ethylene glycol containing 500 mM ammonium sulfate and then concentrated by a DE-52 chromatography, as described above.

Assays for RNA Polymerase II Activity

Assay solutions for non-specific runoff transcription contain, in a total volume of 20 μL, 12 mM Hepes pH 7.9, 60 mM KCl, 3 mM $MnCl_2$, 0.12 mM EDTA, 12 % glycerol, 0.3 mM dithiothreitol, 250 μM each of ATP, CTP, GTP, 25 μM UTP, 0.5 μCi [^{3}H] UTP, and 20 μg/mL of supercoiled DNA plasmid containing adenovirus major late promoter sequence. Transcription in these assays is not promoter dependent. Various volumes of purified RNA polymerase II was also added. Reactions were incubated for 1 hr at 37° C. Then each mixture was spotted onto a DEAE filter disc (DE81, Whatman). The filters were washed with 0.5 M Na_2HPO_4 to remove unincorporated [^{3}H] UTP, then counted using a scintillation counter (Beckman). The standard definition of one unit of enzyme is one μmol product catalyzed by the enzyme per min. However, One unit of RNA polymerase II activity is defined as incorporation of one nmol of UTP into RNA in 10 min.

Preparation of Hela Cell Extracts

Nuclear and DNA binding proteins were extracted from 250 L Hela cells by a modification of the method of Dignam (143). Hela is a human cell culture that was originally derived from a cervical carcinoma. Cells purchased from the National Cell Culture Center (Minneapolis, MN) were cultured in a suspension with Joklik's Spinner Medium containing horse serum. Approximately 1 L of cell culture contains 5 x 10^9 cells and weighs 2 g.

One hundred grams of cells were resuspended with 250 mL of buffer containing 20 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM EDTA, and 0.5 mM dithiothreitol. Then cells were homogenized by Dounce Homogenizer at 4° C. To the homogenate, glycerol and NaCl were added to a final concentrations of 25 % glycerol and 0.5 M NaCl, also Hepes and EDTA were added to compensate for the total volume. After stirring on ice for 20 min, the solution was centrifuged at 30 K rpm for 2 hr in Beckman Ti-45 rotor. The pellet was re-extracted with the same buffer containing 0.6 M NaCl to dissociate DNA binding proteins from DNA. The supernatant was mixed and dialyzed against transcription buffer (20 mM Hepes pH 7.9, 20 % glycerol, 0.1 M KCl, 2.0 mM dithiothreitol, 0.2 mM EDTA, and 0.2 mM EGTA). The dialysate was centrifuged at 35 K rpm for 1 hr. The clear supernatant was stored at -85° C.

Preparation of Affinity Chromatography Columns

Ten mg calf thymus RNA polymerase II purified by ion exchange chromatography from four preparations of one Kg calf thymus was covalently coupled to Affi-Gel 10 (Bio-Rad), and unreacted coupling groups blocked with ethanolamine, as described by Sopta (86). The mixture of 10 mg RNA polymerase II and 5 mL of Affi-Gel 10 was shaken overnight at 4° C and then left for 6 hr in transcription buffer containing 150 mM ethanolamine. The column was washed with transcription buffer containing bovine serum albumin. The control column was prepared identically except that no RNA polymerase II was added in the coupling reaction. Columns were stored at 4° C.

RNA Polymerase II Affinity Chromatography

A 200 mL aliquot of a Hela cell extract was loaded into the control column and then the flow through fraction was loaded into the RNA polymerase II column in transcription buffer containing 0.1 M KCl at 4° C. Columns were washed with 20 mL transcription buffer, then eluted with transcription buffer containing 0.5 M KCl. RAP and control fractions were dialyzed to 0.1 M salt concentration and stored frozen. Columns were used repeatedly to process extracts derived from entire 250 L growth of Hela cells.

Bio-Rex 70 Cation Exchange of Control and RAP Fractions

The control and RAP fractions were further purified by a 100 µl bed weak cation exchanger, Bio-Rex 70 (Bio Rad). The resin was packed in a 2 mm x 1 cm Biocompatible Guard Column Cartridge (Upchurch). The column was previously equilibrated with transcription buffer containing 0.2 mg/ml bovine serum albumin. The dialyzed control and RAP fractions were loaded in separate runs into the column using a peristaltic pump (Pharmacia) at 40 µl/min flow rate. After washing with 1 mL of transcription buffer, the protein fractions were then eluted stepwise with transcription buffer containing 0.3, 0.4, and 0.7 M KCl. Each step elution was collected in 8 - 100 µl fractions.

Microsequencing Analysis

The 0.4 and 0.7 M KCl RAP fractions from Bio-Rex 70 chromatography were pooled and then concentrated by Centricon microconcentrator (Amicon). The concentrated RAP fractions were separated on SDS-PAGE. After staining with Immobilon-CD Stain (Millipore), which is a negative stain originally designed for proteins or peptides immobilized to Immobilon-CD membrane (see operating guide by Millipore), protein bands were cut from the gel and homogenized with 0.1 M ammonium bicarbonate buffer (pH 7.5) containing 6 M guanidine hydrochloride. The solution and homogenized gel fragments were diluted to 1 M guanidine hydrochloride. Trypsin was added directly to homogenized gel slice followed by incubation at 30° C for 24 hr. The peptides obtained from trypsin digestion were separated by a reversed-phase HPLC system (Brownlee) using a C-18 column (1 x 25 mm; Vydac). The peptides were eluted with a gradient buffer from 90 % buffer A (0.1 % trifluroacetic acid) and 10 % buffer B (90 % acetonitrile and 0.08 % trifluroacetic acid) to 100 % buffer B. The sequences of pure peptides were determined by automated Edman degradation with an Applied Biosystems 477A Protein Sequencer and 120A Analyzer in the Michigan State University Macromolecular Structure Facility.

SDS-Polyacrylamide Gel Electrophoresis

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was by the method of Laemmli (144). RNA polymerase II and RAPs were run on a 10 % gel using the Mini-Protean II system (Bio-Rad). RNA polymerase II was stained with Coomassie blue, and RAPs were stained with silver nitrate (145).

Protein Concentrations

Protein concentrations were determined by the method of Bradford (145), using BSA from Pierce Chemical Company as standards.

Western Blot Analysis

After SDS-PAGE, proteins were transferred to Trans Blot (Bio-Rad) nitrocellulose membrane in a solution with 25 mM Tris, 192 mM glycine, and 20 % methanol using the Mini-Protean II system. After blocking with 3 % gelatin in TBS (500 mM NaCl, 20 mM Tris pH 7.5), membranes were incubated with antiserum in appropriate dilutions. Antibodies bound to specific proteins were detected using an appropriate second antibody conjugated to alkaline phosphatase. Blue color was developed using the nitro-blue tetrazolium/bromo-chloro-indoyl phosphate (NBT/BCIP) color reaction according to the manufacturer's instructions (Bio-Rad).

RESULTS :

RNA Polymerase II Preparation

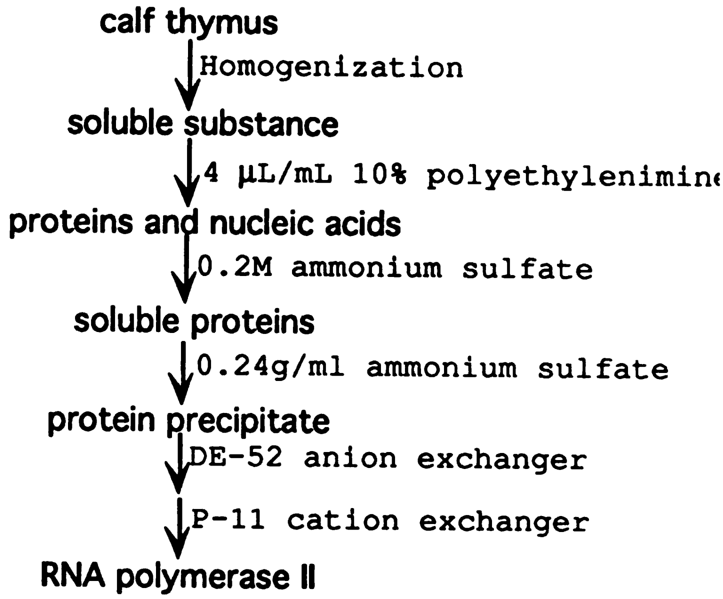
Several preparations of RNA polymerase II have been done to accumulate approximately 20 mg RNA polymerase II. Figure 5(B) shows fractions from preparation steps, and some subunits identified by molecular weights (22) such as II_a (210-kDa), II_b (180-kDa), II_c (145-kDa), II_d (44-kDa), II_e (36-kDa), II_f (25-kDa), II_j (15-kDa), and II_k (12-kDa). The purity of RNA polymerase II obtained by this procedure ranges from 50 to 80 %.

Some of the RNA polymerase II preparations were depleted of II_a form RNA polymerase II by immuno-affinity chromatography on a column containing immobilized anti-CTD monoclonal antibody. RNA polymerase II isolated in this way has the expected subunit structure, subunits II_a, II_b, II_c, II_d, II_e, II_f, II_j, and II_k are identified [Figure 5(C)]. Since neither RNA polymerase I nor RNA polymerase III have a CTD, these polymerases can not bind to an anti-CTD column. Binding to the anti-CTD column, therefore, shows that RNA polymerase II has been isolated rather than RNA polymerase I or III.

RNA polymerase II preparations were tested in a general transcription assay that measures incorporation of isotope from ^{3}H-UTP into RNA using supercoiled plasmid as a template. Since this assay does not contain other transcription factors required for accurate initiation, transcription initiates at many sites on the plasmid rather than from promoters. RNA is separated from unincorporated UTP by binding to DEAE filters and washing with Na_2HPO_4. Each of the RNA polymerase II preparations incorporated UTP into RNA using this assay. One unit of enzyme activity is defined as incorporation of one nmol of UTP in 10 min. One of these assays is shown in Figure 5(D). The specific activity of purified RNA polymerase II is 15 unit/mg. This activity is also sensitive to 1μg/mL α-amanitin, showing that this RNA synthesis is due to RNA polymerase II rather than RNA polymerase I (insensitive to α-amanitin) or RNA polymerase III (sensitive to 200 μg/mL α-amanitin) (21). The specific activity of immuno-affinity purified enzyme was determined to be 97.5 unit/mg. Since only RNA polymerase II, which contains the CTD consensus sequence, can be eluted from the immuno-affinity column, the high specific activity of immuno-affinity purified enzyme shows that anti-CTD column can successfully

Figure 5. Purification of RNA Polymerase II.

(A) Purification scheme.



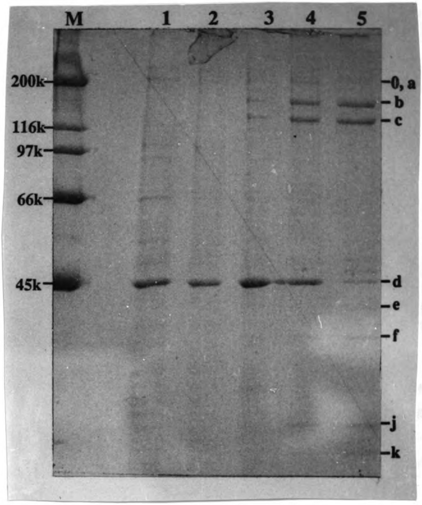


Figure 5 (B) Purification and subunit structure of calf thymus RNA polymerase II. Lane 1) 0.2 M ammonium sulfate extracted protein (4.5 μg). Lane 2) Ammonium sulfate precipitated protein (4.1 μg). Lane 3) DE-52 chromatography peak fraction (4.7 μg). Lane 4) P-11 chromatography peak fraction (3.1 μg). Lane 5) DE-52 concentrated RNA polymerase II (3 μg). M) Protein markers. Some subunits of RNA polymerase II were identified by molecular weights and indicated. This is a 10 % polyacrylamide gel containing SDS stained by Coomassie blue.

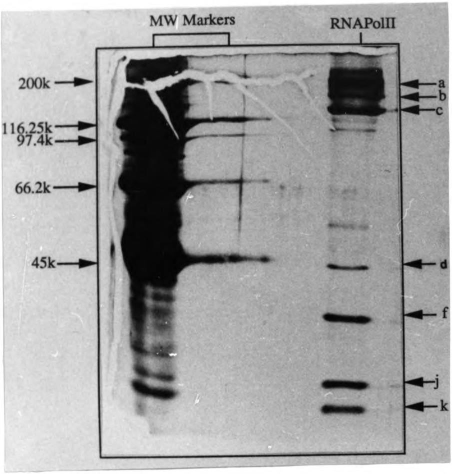


Figure 5 (C) Purification of II_a form RNA polymerase II by anti-CTD column.

II_a form RNA polymerase II was affinity purified as described in methods. Approximately 500 ng of RNA polymerase II purified by anti-CTD chromatography was loaded. Some subunits of RNA polymerase II were identified by molecular weights and indicated. This is a silver stained 10 % SDS - polyacrylamide gel.

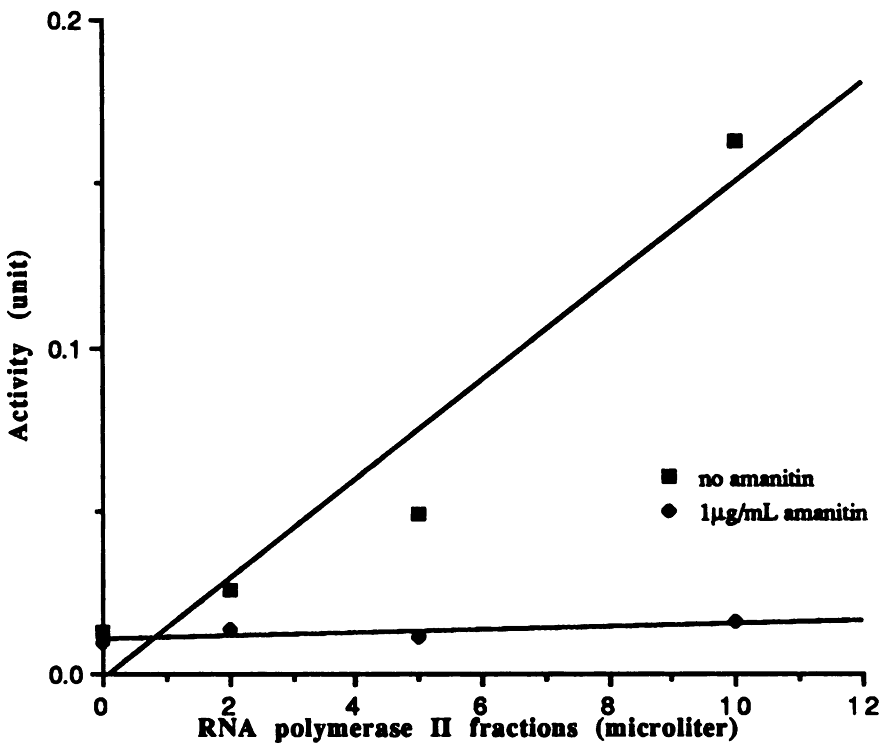


Figure 5 (D) RNA polymerase II activity assay.

Each test was done in duplicate as described in methods. Protein content of this sample is 0.98 mg/ml according to absorbance under UV scan. The calculated specific activity of this sample is 15.3 unit/mg. This enzyme activity is sensitive to 1 μg/ml α-amanitin.

isolate high purity of RNA polymerase II.

Based on the observed subunit structure [Figure 5(B)], the presence of the CTD in this protein [Figure 5(C)], and the α-amanitin sensitivity of the enzyme [Figure 5(D)], it is concluded that the purified protein is RNA polymerase II. This polymerase contains a mixure of II_0, II_a, and II_b forms of the enzyme.

RNA polymerase II that has not been affinity purified has been shown to be suitable for construction of affinity columns for isolation of RAPs (86). Therefore, this material is able to use to construct a RNA polymerase II affinity column.

Isolation of Hela Cell Extracts

Approximately 1 Kg (wet weight) of Hela cells (250 L) were extracted. After cell lysis, nuclei were lysed with 0.6 M NaCl. Nuclear and DNA-binding proteins were extracted and separated from DNA by centrifugation. After dialysis against transcription buffer containing 0.1 M KCl, this extracts are passed through control and RNA polymerase II columns. The volume of extracts from 100 g Hela cells is 400 to 500 ml, and the protein concentration is approximately 2 mg/ml.

RNA Polymerase II Affinity Chromatography

RAPs which are reversibly associated with RNA polymerase II in solution will exchange to the column by binding to immobilized RNA polymerase II. After salt elution, RAPs can be identified on gels by comparing with control fractions [Figure 6] (unpublished). RAPs bind selectively to the RNA polymerase II column. RNA polymerase II chromatography has been successfully used to purify some transcription factors (86). The purified RAP30 and RAP74, which are subunits of TFIIF, have been tested for their requirement in transcription initiation (84). RAP38, which is TFIIS (SII), has been characterized as an elongation factor (127, 128).

Hela cell extracts were processed in 250 mL batches by RNA polymerase II affinity chromatography, as described in methods. The average protein concentrations of control fractions and RAP fractions are 70 μg/ml and 300 μg/ml, respectively.

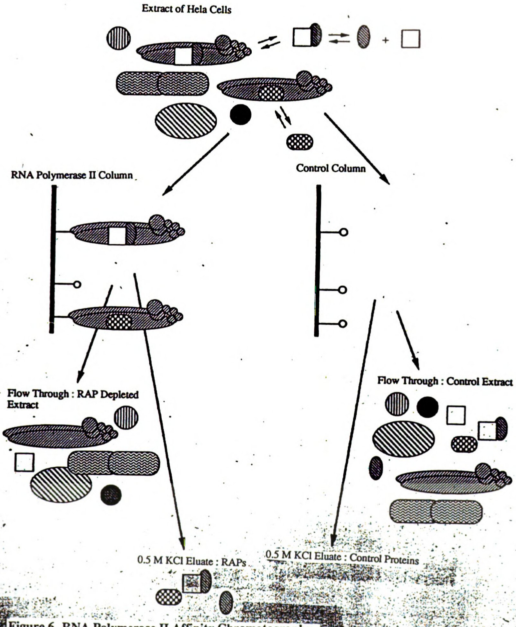


Figure 6. RNA Polymerase II Affinity Chromatography.

By comparing control proteins and RAPs on SDS - polyacrylamide gels, at least ten RAPs were identified. They are RAP110, 87, 74, 69, 50, 38, 34, 32, 30 ,and 25, named by their molecular weights in kDa. In Figure 7(A), which is a 8% polyacrylamide gel showing control and RAP fractions #2 to #4, we identified RAP110, 87, and 69 in fractions #3 and #4, RAP74 and RAP38 in fraction #4, and RAP50 in fractions #2 to #4. In Figure 7(B), which is a 10% polyacrylamide gel showing control and RAP fractions #2 to #5, we identified RAP110, 87, 69, 32, and 30 in fractions #3 to #5, RAP74 in fraction #4, RAP50 in fractions #2 to #5, RAP38, 34, and 25 in fractions #4 and #5. Determined by visually comparing the intensity of protein bands of RAPs with bands of protein markers on silver stained gels, it was obtained, from a run of 250 mL extracts, approximately 50 μg of RAP110, 20 μg of RAP87, 10 μg of RAP74, 20 μg of RAP69, 100 μg of RAP50, 60 μg of RAP38, 5 μg of RAP34, 5 μg of RAP32, 25 μg of RAP30, and 15 μg of RAP25.

Since RAP30, 74, and 38 have been reported in RAP fractions (86), both control and RAP fractions from each batch of RNA polymerase II affinity chromatography were tested by Western blot analysis using specific antibodies against RAP30, RAP74, and RAP38, respectively, to confirm this chromatography is able to successfully isolate proteins associated with RNA polymerase II. Some of these Western blot results are shown in Figure 8.

In Figure 8 (A), three different batches of pooled fractions were tested with anti-RAP30 antibodies. RAP30 is present in RAP fractions but not in control fractions. In Figure 8 (B), three different batches of pooled fractions were tested with anti-RAP74 antibodies. RAP74 is present in RAP fractions but not in control fractions.Recombinant human RAP74 produced in *E. coli* was used as a positive control. The smaller immunoreactive bands in the recombinant RAP74 lane are the result of premature translation termination (146). The smaller proteins present in RAP fractions, and some of control fractions are N-terminal fragments of RAP74 (146). In Figure 8(C), control and RAP fractions #3 to #5 from one batch were tested with anti-RAP38 antibodies. RAP38 is present in RAP fractions #4 and #5, the same as identified on the silver stained gel [Figure 7(B)]. The double bands of RAP38 are different phosphorylated forms (147). Recombinant RAP38 is not phosphorylated, since it is produced in *E. coli*, and therefore does not precisely co-imigrate with human RAP38. Since RAP30, RAP74, and RAP38 are present in RAP fractions but not in control fractions according to the results of Western blot analysis, proteins specifically associated with RNA polymerase II were

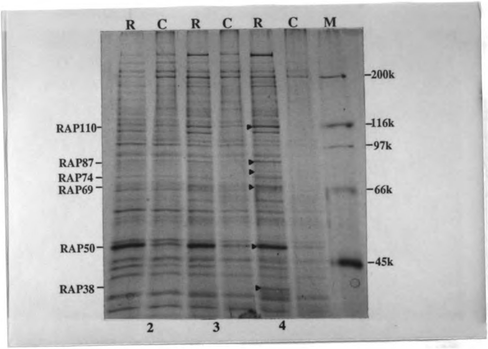


Figure 7. Isolation of Human RAPs by RNA Polymerase II Affinity Chromatography.

(A) A 200 mL aliquot of a Hela cell extract was passed through the control column and then the RNA polymerase II column. After washing, columns were eluted with transcription buffer containing 0.5 M KCl in 8 - 1.5 mL fractions. Fractions #2 to #4 are shown. C) Control fractions (10 μl containing about 0.7 μg total protein); R) RAP fractions (4 μl, 1.2 μg). M) Protein markers (100 ng of each marker). The proteins that bind to the RNA polymerase II column but not to the control column are identified as RAPs. By comparing bands in C and R lanes, several RAPs were identified. Six of these RAPs were named by their molecular weights as indicated. This is a silver stained 8 % SDS - polyacrylamide gel.

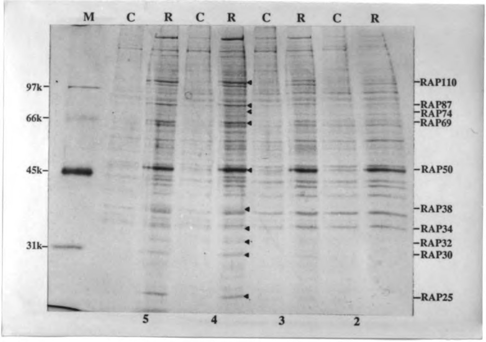


Figure 7 (B) Samples are the same as (A). Fractions #2 to #5 are shown on a silver stained 10 % SDS - polyacrylamide gel. C) Control fractions (10 μl, 0.7 μg); R) RAP fractions (5 μl, 1.5 μg). M) Protein markers (100 ng of each marker). RAP110, 87, 74, 69, 50, 38, 34, 32, 30, and 25 are indicated.

Fig. 8 (A)

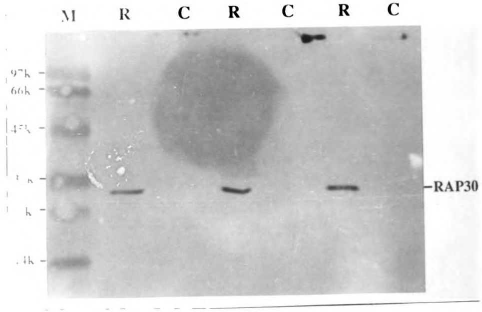


Figure 8. Identification of TFIIF (RAP30/RAP74) and TFIIS (RAP38) in RAP Fractions by Western Blots.

Western blots were developed with : (A) anti-RAP30 antibodies; (B) anti-RAP74 antibodies; and (C) anti-RAP38 antibodies. Three different batches of pooled control and RAP fractions are shown in (A) and (B); fractions #3 to #5 from one batch of control and RNA polymerase II column are shown in (C). C) Control fractions (10 μl containing about 0.7 μg of total protein); R) RAP fractions (5 μl, 1.5 μg). M) Prestained protein markers. In (B), +) 100 ng recombinant RAP74 as positive control. Smaller proteins are N-terminal fragments of RAP74 (146). In (C), +1) 140 ng and +2) 70 ng recombinant RAP38. The double bands shown in R lanes are different phosphorylated forms of RAP38 (147). Antibodies used in these tests were generated in chickens (146 and unpublished data). Second antibodies were rabbit anti-chicken antibodies conjugated to alkaline phosphatase.

Fig. 8 (B)

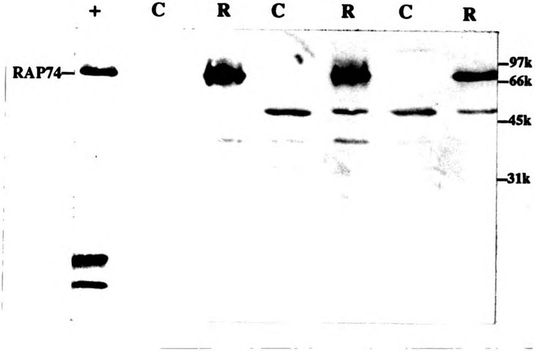
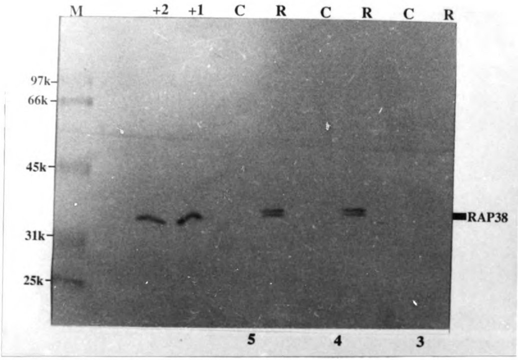

+
C
R
C
R
C
R
RAP74
97k
66k
45k
31k

Fig. 8 (C)


M
+2
+1
C
R
C
R
C
R
97k
66k
45k
31k
25k
RAP38
5
4
3

successfully isolated by this RNA polymerase II affinity chromatography. In addition to RAP30, 74 ,and 38, other RAPs, which may be related to transcription, are targets for further investigation.

TFIIE is found in RAP fractions; TFIIB is not

As described chapter I, RNA polymerase II requires TBP, TFIIB, TFIIE, TFIIF, TFIIH, and TFIIJ, to initiate accurate transcription. However, Buratowski and colleagues showed that TBP, TFIIB, RNA polymerase II, and RAP fractions can reconstitute accurate transcription *in vitro* (97). Therefore, initiation factors TFIIF, TFIIE, TFIIH, and TFIIJ are expected to be in RAP fractions. In addition, TFIIE (97, 108) and TFIIB (77) have been reported to associate stably with RNA polymerase II in solution. Therefore, TFIIE subunits (TFIIE34 and TFIIE56) and TFIIB in RAP fractions were tested by Western blot analysis using specific antibodies.

Figure 9(A) and 9(B) show the results of Western blot analysis for TFIIE subunits. Both TFIIE subunits are present in the positive controls and RAP fractions but not control fractions. Quantitated by visually comparing the intensity with positive controls, the molecular ratio of these two subunits is 1 to 1 in fractions #3 to #5. The positive control proteins are 36 and 58-kDa rather than 34 and 56-kDa because they have extra 10 histidine residues at the N-terminus (103).

RAP32 might be TFIIB (35-kDa) based on its molecular weight. TFIIB, however, is not present in RAP fractions or control fractions basaed on Western blot analysis (data not shown). Since the methods were sufficient to detect a protein of the abundance of RAP34, it is concluded that RAP32 is not TFIIB.

Bio-Rex 70 Chromatography

For concentration and further isolation, both control and RAP fractions were fractionated on a Bio-Rex 70 column. The column was loaded at 0.1 M KCl and eluted with steps of 0.3, 0.4, and 0.7 M KCl buffers. The elution was done in 8-100 μL fractions. Ten RAPs, which are the same as those identified in Figure 7, were identified

Fig. 9 (A)

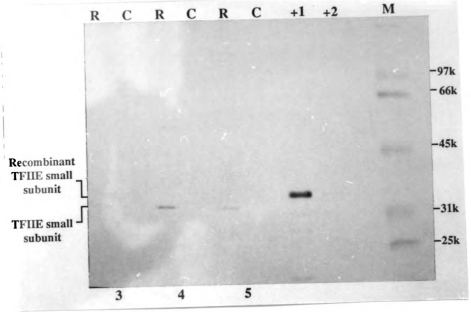
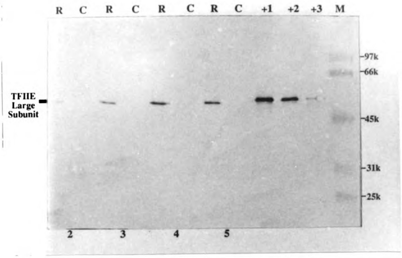


Figure 9. Identification of TFIIE Subunits in RAP Fractions by Western Blots.

Western blots were developed with : (A) anti-TFIIE34 antibodies; (B) anti-TFIIE56 antibodies. Fractions #3 to #5 from control and RNA polymerase II columns are shown in (A). Fractions #2 to #5 are shown in (B). C) Control fractions (10 µl containing about 0.7 µg of total protein); R) of RAP fractions (5 µl, 1.5 µg). M) prestained protein markers. In (A), **+1)** 88 ng and **+2)** 53 ng recombinant TFIIE34. In (B), **+1)** 120 ng, **+2)** 60 ng, and **+3)** 30 ng recombinant TFIIE56. The molecular weights of recombinant proteins are 36 and 58-kDa rather than 34 and 56-kDa because they contain extra 10 histidine residues at the N-terminus (103). Antibodies used in these tests were generated in rabbits (unpublished data). Second antibodies were goat anti-rabbit antibodies conjugated to alkaline phosphatase (Bio-rad).

Fig. 9 (B)



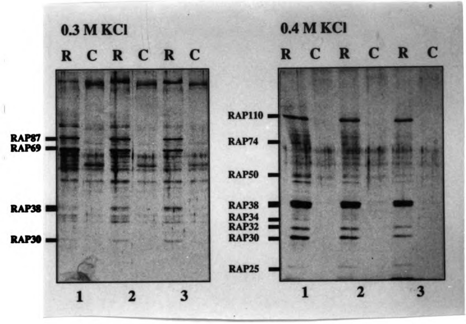
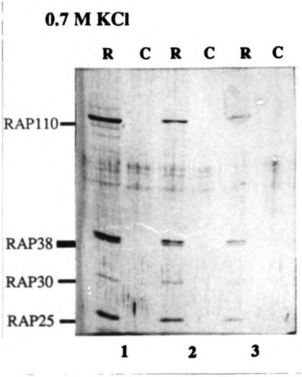


Figure 10. Bio-Rex 70 Cation Exchange Chromatography.

A 100 μl bed Bio-Rex 70 column was loaded separately with control proteins and RAPs in transcription buffer containing 0.1 M KCl. The column was eluted stepwisely with transcription buffer containing 0.3, 0.4, and 0.7 M KCl as indicated. Each elution was done in 8 - 100 μl fractions. The first 3 fractions of each elution are shown. C) Control proteins (5 μl); R) RAP fractions (2 μl). By comparing the protein bands in C and R lanes, many RAPs were identified. The same RAPs identified in Figure 7 are indicated. RAP87, 69, 38, and 30 were identified in 0.3 M KCl fractions. RAP110, 74, 50, 38, 34, 32, 30 and 25 were identified in 0.4 M KCl. RAP110, 38, 30, and 25 were identified in 0.7 M KCl fractions. These are 10 % SDS - polyacrylamide gels stained with silver nitrate.

Fig. 10 (continued)



on SDS-polyacrylamide gels. In Figure 10, the first 3 fractions from the 0.3, 0.4 and 0.7 M KCl elution are shown.

RAP110 was identified in 0.4 and 0.7 M KCl fractions. RAP87 and 69 were identified in 0.3 M KCl fractions. RAP74, 50, 34 and 32 were identified in 0.4 M KCl fractions. RAP38 and 30 were identified in all three salt fractions. RAP25 was identified in 0.4 and 0.7 M KCl fractions. RAP38 has double bands which are different phosphorylated forms, as presented on Western blots in Figure 8(C). RAPs were quantitated by comparing the intensity of RAP bands with protein markers on gels. Each run of 10 to 12 mL of RAP fractions derived from 250 mL extracts was able to obtain approximately 30 μg of RAP110, 15 μg of RAP87, 5 μg of RAP74, 15 μg of RAP69, 20 μg of RAP50, 50 μg of RAP38, 3 μg of RAP34, 3 μg of RAP32, 25 μg of RAP30, and 10 μg of RAP25. The average yield of Bio-Rex 70 chromatography is 63% (Table 2). Use of larger columns causes significant losses of proteins because of non-specific adsorption of proteins to column resins (data not shown).

All three salt fractions were tested by Western blot analysis using specific antibodies against RAP30, 74, 38, TFIIE34, TFIIE56, and TFIIB. Results show that RAP74, 30, 38 were identified in all three salt fractions, and TFIIB was not identified in any fractions (data not shown). TFIIE subunits were identified in 0.4 M KCl fractions only (data not shown).

Microsequencing Analysis

After separation on SDS-polyacrylamide gels and digestion by trypsin, all RAPs are going to be sequenced. So far, a tryptic peptide derived from RAP50 has been sequenced. The sequences are Tyr-Tyr-Val-Thr-Ile-Ile-Asp-Ala-Pro. Searching of nucleic acid and protein sequence databases shows that this peptide matches a fragment of human translation elongation factor-1α (EF-1α) (53-kDa) (193).

RAPs	Bio-Rex 70 Fractions			Yield (μg)		Yield (%)	TFs
	0.3 M	0.4 M	0.7 M	Affinity	Bio-Rex	Bio-Rex	
110		√	√	50	30	60	?TAF120
87	√			20	15	75	?IIH
74		√		10	5	50	IIF
69	√			20	15	75	?IIH
50		√		100	20	20	?
38	√	√	√	60	50	83	IIS
34		√		5	3	60	?
32		√		5	3	60	IIE
30	√	√	√	25	20	80	IIF
25		√	√	15	10	67	?

Table 2. Fractionation and yield of RAPs.

DISCUSSION :

Comparing salt eluates of RNA polymerase II columns and control columns, proteins have been identified that appear to bind specifically to RNA polymerase II. These proteins have been named "RAPs" for RNA polymerase II-associating proteins and have been designated by their apparent molecular weight. The most convincing argument that these proteins are functionally associated with RNA polymerase II can be made when they are demonstrated to alter the transcriptional activity of RNA polymerase II. This criterion has been met for three RAPs, RAP30, RAP74, and RAP38. RAP30/74 has been shown to be transcriptional initiation and elongation factor TFIIF (85). This factor is required to bring RNA polymerase II into the preinitiation complex (42). The RAP74 subunit of this factor has been shown to be required for RNA polymerase II to escape from a promoter after initiation (100). RAP38 is an elongation factor for transcription by RNA polymerase II (127).

Using Western blot analysis, it is shown that both TFIIE subunits are present in RAP fractions but not in control fractions (Figure 9), so both TFIIE subunits are RAPs. This result corresponds to an earlier report showing that TFIIE is able to associate stably with RNA polymerase II in solution (97, 108). RAP34 is likely to be the small subunit of TFIIE, TFIIE34, because of following reasons. First, RAP34 has a similar SDS-PAGE mobility as the small subunit of TFIIE presented on Western blot membrane. Second, RAP34 is only identified in 0.4 M KCl fractions from Bio-Rex 70 chromatography (Figure 10), whereas Western blot analysis has shown that both TFIIE subunits is present only in the same salt fractions as RAP34 (data not shown). We were not able to identify a RAP that corresponds to TFIIE56, since it appears that there is a control protein with a similar SDS-PAGE mobility as TFIIE56.

Numerous other RAPs have been tentatively identified including RAPs of 110, 87, 69, 50, 34, and 25-kDa. Until the function of these proteins in transcription is established, the importance of their binding selectively to an RNA polymerase II column will not be known. For instance, a protein might bind to a contaminant of the RNA polymerase II preparation rather than to RNA polymerase II. This problem can be circumvented by demonstrating binding between RNA polymerase II and RAPs in a glycerol gradient. Because of the large size of RNA polymerase II, RAPs would not co-migrate with polymerase unless they were specifically bound.

In some cases, a control column binding protein co-migrate with a suspected RAP by SDS-PAGE, although the intensity, and sometimes the color, of the silver-stained protein is different. The question then arises of whether these are the same or different proteins. Further separation of proteins on Bio-Rex 70 in most cases shows that control proteins and RAPs of similar sizes elute in different Bio-Rex 70 fractions, indicating that the control protein is a different protein from the RAP with different properties. When antibodies become available for specific RAPs, Western blots of control and RAP fractions can be done. If the antibody reacts with a protein in the RAP fraction but not in the control fraction, this experiment confirms that the RAP binds selectively to the RNA polymerase II column.

Some of these RAPs have similar molecular weights as previously identified transcription factors. RAP110 might be a TAF (TAF120 or 100) (62), or an initiation factor TFII-I (120-kDa) (195). RAP87 and RAP69 might be 90-kDa (85-kDa from rat liver) and 62-kDa (68-kDa from rat liver) subunits of TFIIH (106, 109, 110). This argument is supported that they are co-eluted with 0.3 M KCl from a Bio-Rex 70 column. RAPs with similar molecular weights as other three subunits of TFIIH (43, 40, 35-kDa) were also identified in the 0.3 M KCl fractions as minor bands on gels.

It has been reported that TFIIB associates stably with RNA polymerase II *in vitro* (77). We considered the possibility that RAP32 might be TFIIB (35-kDa) based on its molecular weight. However, TFIIB is not present in RAP fractions based on Western blot analysis (data not shown). There are two possibilities : (a), TFIIB binds to RNA polymerase II very tightly; and (b), human TFIIB binds to human RNA polymerase II specifically. Therefore, TFIIB can not exchange from free RNA polymerase II in extracts to immobilized RNA polymerase II.

Although it is possible RAP32 is the 35-kDa subunit of TFIIH, it is not likely to be the case because it is not coeluted with RAP87/69. These RAPs can be tested by Western blot analysis when we obtain specific antibodies from other laboratories.

A tryptic peptide derived from RAP50 has been sequenced. Surprisingly, searching of nucleic acid and protein sequence databases shows that RAP50 is human translation elongation factor-1α (EF-1α) (53-kDa) (193). This factor is involved in binding of aminoacyl-tRNA to 80S ribosomes. During this process GTP is hydrolyzed into GDP. In order to perform this function, EF-1α has domains that bind guanine nucleotides, 80S ribosomes and aminoacyl-tRNAs. In addition, EF-1α interacts with EF-1β to exchange bound GDP to GTP (194). It is not likely that translation elongation factors are involved in the transcription process, since transcription and translation events in eukaryotic cells are separated by the nuclear membrane. It is possible that EF-1α associates with some contaminant of the impure RNA polymerase II. Further evidence is required to demonstrate whether EF-1α is a RAP and has function in transcription process.

Establishing a functional assay for a RAP in transcription can present some difficulties. For RAP30/74, this was accomplished by immunoprecipitating the RAP30/74 complex using anti-RAP30 antibodies (91). This RAP30/74-depleted extract was inactivated for accurate transcription activity. The depleted extract was then reconstituted by addition of recombinant human RAP30 and RAP74. But not all transcription factors are required initiation factors. In some cases, the appropriate functional assay for a RAP may not yet be established. If a RAP were involved in splicing and polyadenylation in termination of transcription, for instance, the appropriate functional assay for this RAP will require a significant amount of development. With this in mind, our laboratory has initiated studies of RAPs in brewer's yeast *S. cerevisiae*. If a human RAP is found to have a yeast homolog, yeast genetics can be applied to identify a function for that RAP in yeast cells.

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