A single cDNA, hTFIIA/ α , encodes both the p35 and p19 subunits of human TFIIA

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TFIIA is a transcription factor that, by interacting with the TATA-binding subunit (TBP) of TFIID, modulates transcription initiation by RNA polymerase II *in vitro*. By use of a mobility shift assay, TFIIA was purified from HeLa cells as a complex of 35-, 19-, and 12-kD subunits. Oligonucleotides were used to isolate a human cDNA clone, hTFIIA/ α , which encodes a 55-kD protein with homology to the product of the yeast gene TOA1. The open reading frame of hTFIIA/ α contains peptide sequences obtained from both the p35 and p19 subunits of natural human TFIIA, and thus encodes these two subunits. Consistent with this, antiserum raised against the 55-kD hTFIIA/ α -encoded protein reacted with both the p35 and p19 subunits of natural TFIIA, and the recombinant protein could functionally replace those subunits in a mobility shift assay with renatured p12. An efficient affinity purification for natural human TFIIA was suggested by the sequence of the hTFIIA/ α protein and demonstrated biochemically. Finally, transcription from the adenovirus major late promoter was greatly reduced in nuclear extracts depleted with anti-TFIIA/ α serum and was restored to original levels by the readdition of purified human TFIIA.

[**Key Words:** Transcription factor IIA; purification; cloning; transcription]

Received March 12, 1993; revised version accepted August 3, 1993.

Transcription initiation by RNA polymerase II requires a number of basic factors, including TFIIA, TFIIB, TFIID, TFIIE, TFIIF (for review, see Roeder 1991; Zawel and Reinberg 1992), TFIIG/J (Sumimoto et al. 1990; Cortes et al. 1992), and TFIIH (Flores et al. 1992). The first step in the ordered assembly of these factors into a preinitiation complex is the binding of the TATA-binding subunit (TBP) of TFIID to the TATA box of the promoter in a step that may be facilitated by TFIIA (Reinberg et al. 1987; Buratowski et al. 1989). Although they are not essential for basal transcription, other cofactors (Meisterernst et al. 1991) and TBP-associated proteins (TAFs) within TFIID (Hoffmann et al. 1990; Pugh and Tjian 1990; Dynlacht et al. 1991; Tanese et al. 1991; Zhou et al. 1992) are required for activation of transcription by upstream factors. Many of these general initiation factors and cofactors have been cloned and shown to be functional, and soon it should be possible to study transcription in a completely defined system.

TFIIA was identified originally as a HeLa cell-derived phosphocellulose flowthrough fraction with transcriptional stimulatory (Matsui et al. 1980; Samuels and Sharp 1986) and TBP-dependent mobility shift (Buratowski et al. 1989) activities. Further purification of TFIIA from mammalian sources revealed variable subunit compositions, consisting of 19- and 12-kD polypeptides (Samuels and Sharp 1986), a single 35-kD polypeptide (Usuda et al. 1991; Waldschmidt and Seifart 1992), or 35-, 19-, and 12-kD polypeptides (Cortes et al. 1992; Coulombe et al. 1992). Recently, TFIIA was purified

from yeast and shown to consist of p43 and p12 subunits (Ranish and Hahn 1991). Two essential yeast genes encoding these subunits were cloned and the corresponding recombinant proteins shown to enhance transcription in reconstituted yeast and, to a lesser extent, mammalian systems (Ranish et al. 1992). Until now, however, none of the human TFIIA subunits has been cloned.

The reconstituted transcription systems employed in different laboratories have not shown a consistent requirement for TFIIA, and so the role of this factor has remained elusive. Indications of TFIIA function in activator-dependent transcription, however, have come from the recent discovery of TBP-interacting protein, which could compete with TFIIA for TBP binding and, in doing so, direct TBP toward functional or nonfunctional complexes (Meisterernst and Roeder 1991; Meisterernst et al. 1991). Recently, a clone was isolated that encodes a protein, Dr1, that possesses the properties expected of these negative factors (Inostroza et al. 1992). In addition, a number of activator proteins, including VP16 (Stringer et al. 1990), Zta (Lieberman and Berk 1991), E1A (Lee et al. 1991), p53 (Truant et al. 1993), PU.1 (Hagemeier et al. 1993), and CCG1 (Takada et al. 1992; Kokubo et al. 1993) have been shown to directly interact with isolated TBP. This suggests that there will be a complex interplay of factors that bind to TBP. Whether TFIIA bound to TBP will facilitate or prevent simultaneous interactions with upstream factors is not known and may well depend on the nature and timing of these interactions. Thus, whereas TFIIA was shown to be required for high-level

induction by Sp1 in a reconstituted system, preincubation of TFIIA with DNA and general factors appeared to interfere with induction in the presence of the upstream stimulating activity (USA) cofactor fraction (Meisterernst et al. 1991). On the other hand, rapid transition from a closed to an open promoter complex required preincubation of Gal4-AH, TFIID, and TFIIA, suggesting that these three factors take part in some rate-limiting step during activation (Wang et al. 1992). Whether steric competition for TBP interactions will turn out to be the principal mechanism of TFIIA action is not known, but it is likely that TFIIA might itself possess separate protein-protein interaction domains, which function by associating either with upstream activators or basal transcription factors.

Here, we report the purification of TFIIA as a three-subunit complex (p35, p19, and p12) from HeLa cells, and the isolation of a human TFIIA cDNA (hTFIIA/α), which encodes both the p35 and p19 subunits. The 55-kD cDNA-encoded recombinant protein interacts with the natural p12 subunit to generate a characteristic TFIIA-TBP mobility shift. Antibodies raised against the recombinant protein react specifically with the two larger TFIIA subunits and can supershift the natural TFIIA-TBP-promoter complex. Moreover, these antibodies

have been used in immunodepletion and natural TFIIA readdition assays to demonstrate the function of TFIIA in transcription from the adenovirus major late (AdML) promoter. In addition, the deduced sequence predicts an extremely efficient human TFIIA affinity purification scheme, which we demonstrate biochemically.

Results

Purification of TFIIA

By use of a TBP-dependent mobility shift assay (Buratowski et al. 1989) purification of TFIIA from HeLa cell cytoplasmic extracts was accomplished in six chromatographic steps (Fig. 1A,B), the first three of which were based on earlier published results (Matsui et al. 1980; Samuels and Sharp 1986; Reinberg et al. 1987). The putative TBP-TFIIA complex could be further supershifted by the addition of purified TFIIB (data not shown), confirming the identity of the TFIIA shift (Buratowski et al. 1989; Maldonado et al. 1990). Hydroxylapatite provided the first significant purification step (~10-fold), and presumably separated TFIIA from the TFIIF activity reported earlier (Cortes et al. 1992). Chromatography of TFIIA on HPHT-HPLC (or DEAE-5PW HPLC) often resulted in

Figure 1. Purification of TFIIA from HeLa nuclear extract. (A) Schematic of the chromatographic steps used to purify TFIIA. Except for the HAP and HPHT-HPLC hydroxylapatite steps, in which TFIIA was eluted with KPi, the numbers refer to molar concentrations of KCl. (B) Silver-stained SDS gel of consecutive chromatographic steps (lanes 2-7) in the purification scheme shown in A. Molecular weight markers and TFIIA subunit sizes are indicated in kilodaltons (kD). (C) TFIIA-TBP mobility shift analysis illustrating the upper (U-TFIIA) and lower (L-TFIIA) forms as observed after HPHT-HPLC column chromatography (lanes 2-8 are from column fractions 36-42) (D) TFIIA-TBP mobility shift activity of the DEAE-5PW fraction passed over an Affigel-yTBP affinity column. Input, flowthrough (F.T.), and steps of increasing salt concentrations are indicated at the top of the gel. (E) Twice silver-stained SDS-PAGE of the final (0.7 M KCl) TFIIA preparation (lane 2) with yTBP affinity column salt steps shown in the scheme in A. Markers and TFIIA subunit sizes are shown at either side.

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overlapping fractions giving two distinct shifts: a slower-migrating mobility shift species (U-IIA), which generally eluted earlier, and a faster-migrating bandshift species (L-IIA), which eluted at slightly higher salt concentrations (Fig. 1C). DEAE-5PW HPLC chromatography of either U-IIA or L-IIA resulted in the recovery of several mobility shift complexes, the predominant species being L-IIA. The relative levels of these complexes were variable in different experiments and may reflect either the formation of TFIIA multimers, consistent with the suggestion of Usuda et al. (1991), or conformational variants determined by the composition of particular fractions. Because mixtures of chromatographically separated U-TFIIA and L-TFIIA showed mostly the U-IIA mobility shift (data not shown), however, it is possible that a subunit of TFIIA, or interacting factor(s), were partially lost during the purification.

Because the stability of TBP-TFIIA-DNA complexes in mobility shift assays (Buratowski et al. 1989) suggested the possibility of stable TFIIA-TBP interactions, a final protein-protein affinity step with recombinant TBP coupled to Affigel-10 was employed. This approach was taken after previous efforts to affinity-purify TFIIA on Ni-resin columns loaded with His-tagged TBP had failed. As shown in Figure 1D, the coupled resin retained all of the TFIIA mobility shift activity, although the capacity for TFIIA was very low ($\sim 1 \mu\text{g}/\text{ml}$ of resin), perhaps because a heterologous TBP was used. Because of this, large quantities of TFIIA could be obtained only by pooling eluates from multiple column runs. Three bands, p35, p19, and p12, were consistently coeluted from this column (Fig. 1E) and in constant amounts relative to one another, suggesting that all three copurify as a single complex. Interestingly, the 19-kD subunit often stained negatively with high developer concentrations, a property reminiscent of that of the yeast TFIIA large subunit (Ranish and Hahn 1991). Coomassie blue staining of the three subunits following transfer to poly(vinylidene difluoride) (PVDF) membranes, however, revealed roughly equal intensities. The final yield of each band was estimated to be $\sim 5 \mu\text{g}$ from nearly 15 g of starting material. Amino-terminal sequence determinations were attempted, but all three subunits had modified or blocked termini. Therefore, microsequencing of endoproteinase LysC-digested peptides was performed, and four sequences (P1-P4) were obtained from the p19 subunit. To substantiate and extend these results, a subsequent large-scale preparation of TFIIA was prepared by use of the affinity purification described below. The p19 subunit from this new preparation yielded two of the same peptides (P3, P4) that we had identified previously, substantiating the earlier results, whereas the p35 subunit yielded a new peptide P5 (see legend to Fig. 2 for peptide sequences).

Cloning of hTFIIA/ α , a human TFIIA subunit cDNA

Two of four peptide sequences (P3 and P4) could be aligned to the carboxy-terminal region of the yTFIIA subunit TOA1 (Ranish et al. 1992). By combining them, a

fourfold degenerate 63-mer oligonucleotide probe containing the neutral base inosine was designed. Approximately 1×10^6 plaques from two separate Namalwa (NAM) cell λ ZAP libraries were screened with this probe, and each screening yielded a single positive signal, one of 1.1 kb (λ 17-1) and another of ~ 2.2 kb (λ 15-1). Both cDNA clones were incomplete at the 5' end, based on sequence comparisons to the TOA1 gene. Clone λ 17-1 was oligo(dT) primed at an internal A-rich tract just downstream of the putative stop codon, and λ 15-1 was probably primed at a similar tract farther downstream because no poly(A) addition signal was found. By use of the 1.1-kb λ 17-1 *Eco*RI insert as a probe, 1×10^6 plaques of a third random-primed BJA-B λ ZAP library were screened, and three clones were isolated. One (λ 11) was an apparently full-length 2.2-kb clone, and another (λ 6) was primed from within the open reading frame, but contained ~ 300 additional nucleotides upstream of the putative ATG. All of the clones isolated were apparently derived from a single mRNA species, although the relative locations of their 5' and 3' ends were different. On the basis of these isolates, the composite length of an intact cDNA clone (hTFIIA/ α) is expected to be ~ 2500 nucleotides (see Discussion). The predicted amino acid sequence is 376 amino acids (Fig. 2), nearly 100 amino acids longer than the TOA1-encoded yeast subunit, and specifies a protein with a calculated molecular mass of 41.5 kD and an isoelectric point (pI) of 4.2. Three of the peptide sequences obtained from the p19 subunit (P2-P4) were present at the carboxyl terminus of the deduced sequence. A sequence that appears to correspond to that of the P1 peptide is located at the amino terminus, and

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MANSANTNTVEFKLYRSVIEDVINDVRDIFLDDGVDEQVLMELKTLWENKL 50
P1
MQSPAVDGFHSEEQQLLLQVQQQHQPOQQTCHHHHHHHHQAQPOQTPVQCA 100
P5
QTQQVLIPASQQATAPQVIVPDSKLIQHNMASNMASAAATAATLALPAGVT 150
PVQQCILTNSGQLLQVVRANGAQYIFQPPQSVVLQQQVIPQMPPGGVQAP 200
VIQQVLAPLFGGISPPQTGVIIQPOQILFTGNKTQVIPTTVAAPTQAQAI 250
TATGQQQPQAQPAQTQAPLVLVQVDGTGDTSSSEDEDEEDYDDDEEDKE 300
KDGAEQGVVEEPLNSEDDVSDDEEGQELFDTENVVVCQYDKIHRSKNKKK 350
P2 P3 P4
FHLKDGIMNLNGRDYIFSKAIGDAEW 376

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Figure 2. Deduced amino acid sequence of the hTFIIA/ α subunit cDNA. Shown is the predicted protein sequence using the single-letter amino acid code. The location of the peptides (P1-P5) obtained by microsequencing are overlined. The unique polyhistidine and acidic regions are underlined. The peptide sequences obtained from the first TFIIA preparation (see text) are P1-LXENK, P2-F/sHLK, P3-GIMNLNGRDYIFsK, and P4-IG-DAEW, all of which are from the p19 subunit. A subsequent TFIIA preparation yielded (P3)-D/gGIMNLNGRDYIFSK and (P4)-AIGDAE, both from the p19 subunit, as well as (P5)-VD/fG/eFH/mSEEQQLLLQVQQQXQP from the p35 subunit. Lowercase letters in these peptides refer to minor identified amino acids while X denotes unknown residues.

while ostensibly from the p19 subunit, requires further authentication. In addition, the peptide sequence (P5) obtained from the p35 subunit was present between amino acids 56 and 76 (Fig. 2). The assignment of the initiating methionine is based on an appropriate context for the initiation of translation (Kozak 1984), homology to the yeast TFIIA TOA1 gene, and the presence of in-frame upstream stop codons.

The amino acid sequence is remarkable in several respects. First, comparison of the sequence to the analogous yeast gene TOA1 allowed division into four distinct regions (Fig. 3A,B). The first 59 amino acids (region I) are 63% similar between the two species. Region IV consists of 45 amino acids that are 71% similar and is located at the carboxyl terminus in both species. Region III is highly acidic and contains within it a cluster of amino acids (residues 282–298) consisting of 16 consecutive aspartate or glutamate residues broken only by a single tyrosine. There is a corresponding region in TOA1 just upstream of the carboxyl terminus (residues 169–188 and 221–241), and although the primary amino acid sequence appears to have been shuffled between the two species, both proteins have retained very high overall acidity in this region. Interestingly, region II, which

comprises more than half of the hTFIIA/ α sequence, appears entirely distinct from the yeast TOA1 sequence.

Second, there is a decidedly nonrandom distribution of certain amino acids (Fig. 3A). Glutamine accounts for 25% of the residues in region II, although clusters between 64 and 104, 173 and 204, and 247 and 272 stand out. Amino acids 81–87 are consecutive histidines, flanked largely by glutamines. This histidine cluster immediately raised the possibility of using a nickel chelate column (Hochuli et al. 1987) as an efficient single-step affinity purification method (see below). Proline is present in region II at a level of 12%, contrasting with regions I, III, and IV, which together contain only three other proline residues. Moreover, the high proline content suggests the possibility that region II might have a relatively disordered structure. Region III is notable mostly for its preponderance of acidic residues and is followed by a cluster of basic amino acids similar to that found in TOA1. Although it is not an exact match to a consensus nuclear localization signal, this cluster might serve as such and/or be involved in weak interactions with DNA.

A hydrophilicity plot (Fig. 3C) highlights the histidine cluster and the acidic region III. A plot predicting sec-

Figure 3. Characteristics of the hTFIIA/ α sequence. (A) Diagrammatic representation of the predicted amino acid sequence (1–376) is shown by the central bold line marked at 100-amino-acid intervals. Above, the hTFIIA/ α sequence is divided into four general regions, I, II, III, and IV. I and IV are similar to the yeast TFIIA gene TOA1 to the extent indicated, whereas region III is a region rich in charged aspartic acid and glutamic acid residues also comparable to that found in TOA1. Region II is apparently unique. Below is a diagram of the distribution of selected amino acids in the hTFIIA/ α protein. (B) Homologies between the hTFIIA/ α protein and the yeast gene TOA1. The upper sequence is from the hTFIIA/ α sequence; the lower sequence is from the yeast TOA1 gene. The top alignment compares region I of hTFIIA/ α (residues 1–59) with the amino terminus of TOA1 (residues 1–54). The lower alignment compares hTFIIA/ α region IV (residues 331–376) with the carboxyl terminus of TOA1 (residues 240–286). Asterisks (*) indicate identity; carats (^) show similarity. (C) Hydrophathy plot of the hTFIIA/ α amino acid sequence by the method of Kyte and Doolittle. The vertical scale represents relative hydrophilicity (above 0.00) and relative hydrophobicity (below 0.00). The x axis represents the linear amino acid sequence marked at 50-amino-acid intervals. (D) Helix (top line), sheet (middle line), and turn (bottom line) secondary structure predictions. Shaded boxes on a

particular line indicate regions with structures predicted both by Chou–Fasman and Robson–Garnier analysis. The x axis is the linear amino acid sequence.

ondary structure (Fig. 3D) shows potential α -helical regions near the amino terminus and in regions III and IV, and possible β -sheet/turn motifs in region II. Interestingly, amino acids 17–45 almost exclusively alternate hydrophobic residues with acidic aspartic acid and glutamic acid residues. Searches of the Genbank data base selected those proteins with high glutamine content, particularly those with polyglutamine regions, but these sequence similarities did not appear to be specific. Separate searches with subdivisions of the protein did not reveal convincing homology to any proteins other than TOA1.

Expression of hTFIIA/ α

Initial expression of amino acids 129–376 as a histidine-tagged fusion (pET11d129-376) in *Escherichia coli* resulted in the appearance of a 40-kD protein (Fig. 4A, lane 2), whereas a full-length (pET11aN1-376) encoded protein migrated at ~55 kD, the extra 15 kD roughly consistent with the addition of the extra 129 amino acids (Fig. 4A, lane 4). This apparent 55-kD full-length size was surprisingly different, both from the predicted size (41.5 kD) and from the size of any of the purified TFIIA subunits. The sum of the sizes of both the p19 and p35 subunits (whose peptide sequences were present in the deduced sequence), however, is nearly 55 kD. Interestingly, expression of pET11aN1-376 was often accompanied by the production of numerous smaller p55-derived proteins (J. DeJong and R.G. Roeder, unpubl.), and we are currently investigating their origin. To verify that the

full-length expression construct could initiate and terminate translation as expected, regions spanning the initiating methionine and the termination codon were confirmed by sequencing.

To see whether the original λ 11 isolate could encode a 55-kD protein in reticulocyte lysates, we expressed a pB-luescript II plasmid containing this insert (pBShTFIIA/ α). Consistent with the results in bacteria, a [35 S]methionine-labeled protein of 55 kD was observed (as well as a few smaller bands) in a coupled transcription-translation system (Fig. 4B, lane 1). This confirmed our assignment of the initiating methionine and also suggested that the TGA termination codon is used as expected in both the bacterial and in reticulocyte lysate expression systems. Truncation of the template with *MscI*, *AlwNI*, and *PvuII* lowered the mobility of the labeled polypeptides to 30, 28, and 20 kD, sizes that corresponded approximately to the number of amino acids remaining in these deletions (Fig. 4B, lanes 2–4). These data indicate that the observed migration is higher than the predicted size, which is partly attributable to carboxy-terminal sequences and possibly the acidic region III.

Antibodies raised against hTFIIA/ α react with purified TFIIA

To gain an understanding of the relationship between recombinant p55 and the p35, p19, and p12 subunits of purified TFIIA, and to ascertain possible functions of TFIIA in transcription, we generated polyclonal antisera. Sera were initially raised against amino acids 129–376

Figure 4. Expression of the hTFIIA/ α subunit cDNA. (A) Western blot of bacterially expressed full-length and amino-terminal-truncated TFIIA/ α proteins by use of α 129-376 antiserum. Lanes 1 (uninduced) and 2 (IPTG induced) are from cultures containing pET11d129-376. Lanes 3 (uninduced) and 4 (IPTG induced) are from cultures containing pET11aN1-376. Protein size markers are shown in kilodaltons (left). Below is a schematic of regions of the hTFIIA/ α protein, which were expressed as His-tagged fusions for the production of antibodies (see Materials and methods). (B) Coupled transcription-translation expression of hTFIIA/ α . Shown is an autoradiograph of [35 S]methionine-labeled proteins from reticulocyte lysates programmed with full-length (lane 1) or restriction enzyme-truncated pBShTFIIA/ α templates (lanes 2–4) as diagramed below. Size markers (kD) are indicated at left.

(α 129-376), and subsequently against the full-length p55 (α 1-376). In addition, antisera were raised against selected subregions of the clone (residues 1-129 and 304-376; see Fig. 4A). To show the usefulness of these sera as probes for functional studies, we assessed cross-reactivity in solution. Increasing amounts of the α 129-376 serum were coincubated in a γ TBP-TFIIA mobility shift assay and resulted in the appearance of an antibody-dependent supershift (Fig. 5A, lanes 2-7), which was not observed with preimmune serum (Fig. 5A, lane 8). Importantly, these results demonstrate that serum against the 55-kD recombinant protein is specific to natural TFIIA.

To assess further the relationship between the hTFIIA/ α and purified TFIIA, a Western blot analysis was performed. The α 1-376 sera collected after the first boost showed strong cross-reactivity against the 35-kD subunit and, under optimal conditions, very weak cross-reactivity to the p19 subunit (Fig. 5B, lane 1). Because the p19 subunit was not reliably detected, probably attributable to a combination of inefficient transfer as well as weak immunogenicity, α 129-376 serum collected following a third boost of antigen was used in a similar

analysis. In this experiment, substantially stronger reactivity with the p19 subunit was observed (Fig. 5B, lane 2), supporting the idea that the p35 and p19 subunits are both derived from the hTFIIA/ α -encoded protein. The reactivity of the p19 subunit with the α 129-376 serum is consistent with the observation that peptide sequences from this subunit (P2-P4) were located toward the carboxyl terminus of the p55 protein. No reactivity to a specific p55 protein has yet been observed in these analyses.

Recombinant p55 substitutes for the 35- and 19-kD subunits of natural TFIIA

To examine whether a functional correspondence existed between the recombinant p55 protein and the 35- and 19-kD subunits of TFIIA, mobility shift analysis was performed with renatured proteins. For this purpose, an affinity-purified TFIIA preparation (see Fig. 6B, lane 4) was transferred to an Immobilon membrane after SDS-electrophoresis, and the 35-, 19-, and 12-kD regions were excised and renatured [Szewczyk and Summers 1988]. As shown in Figure 5C, none of the renatured TFIIA sub-

Figure 5. Antiserum against hTFIIA/ α recognizes hTFIIA and recombinant p55 can substitute for the p35 and p19 subunits in forming a TFIIA-dependent mobility shift complex. (A) Antiserum against p55 can specifically supershift a γ TBP-hTFIIA complex. All lanes contain DEAE-5PW TFIIA, γ TBP, and an AdML TATA-containing oligonucleotide probe. Lanes 2-7 contain increasing amounts of hTFIIA/ α antiserum (lane 2, 1 μ l, 1 : 100 dilution; lane 3, 1 μ l, 1 : 10; lane 4, 1 μ l, 1 : 5; lane 5, 1 μ l, 1 : 2; lane 6, 1 μ l; lane 7, 2 μ l). Lane 8 is a reaction incubated with 1 μ l of preimmune serum (PI). The γ TBP-TFIIA complex is indicated by D/A. (B) Detection of TFIIA subunits by Western blotting. The Ni-resin-purified TFIIA fraction (15 μ l) was cross-reacted with α 1-376 antiserum collected after one boost (lane 1), or with α 129-376 antiserum collected after the third boost (lane 2). The cross-reactive p35 and p19 subunits are indicated at right. (C) Mobility shift analysis of various combinations of recombinant p55 with isolated and renatured TFIIA subunits. The control (lane 11, 0.1 μ l) is Ni-resin-purified TFIIA. Approximately 100 ng of Ni-resin-purified recombinant p55 (1 μ l) and 2 μ l of each renatured component were used as indicated above the autoradiograph. In addition, lanes 9 and 10 contain 2 μ l of either preimmune or immune serum (α 1-376), respectively. The positions of the shifted complexes and the free probe are indicated at right.

units resulted in a supershift when assayed individually (lanes 2–4). Not surprisingly, the bacterially expressed p55 species also showed no mobility shift activity (lane 5), consistent with the results of independent TOA1 expression and analysis (Ranish et al. 1992). Only when recombinant p55 and renatured p12 were coincubated did a TBP-dependent supershift appear (lane 8). Coincubation of immune serum, but not preimmune serum, abolished the shift (lanes 9,10), demonstrating the presence of p55 (or p55 fragments) in the complex. Importantly, the p55/p12-dependent mobility shift was identical in mobility to the natural TFIIA-dependent mobility shift (lane 11). Moreover, coincubation of the excised and renatured TFIIA subunits resulted in a mobility shift only when p35, p19, and p12 polypeptides were each present (lane 15), consistent with the results of Cortes et al. (1992). This mobility shift was identical to that observed when recombinant p55 was coincubated with renatured p12 (lanes 8,15), demonstrating that p55 can functionally substitute for the two larger TFIIA subunits. These results provide additional biochemical evidence that both p35 and p19 are derived from the hTFIIA/ α -encoded p55 protein.

Affinity purification of natural TFIIA by Ni-resin

The presence of an intrinsic histidine tag in the hTFIIA-deduced sequence suggested that a rapid and efficient purification of hTFIIA might be possible. We already knew that the recombinant p55 protein, expressed in bacteria without any vector-encoded affinity tag, was capable of being purified on Ni-resin (Hochuli et al. 1987) under denaturing and nondenaturing conditions (data not shown). We therefore sought to demonstrate this property with natural TFIIA and to use this approach to assess the subunit composition of TFIIA when purified rapidly from nuclear extracts, rather than after a lengthy purification regimen. Nuclear extract was passed directly over a Ni-resin column, step-eluted with successively higher concentrations of imidazole (20, 30, 40, and 100 mM), and assayed for yTBP-dependent mobility shift activity. As shown in Figure 6A, imidazole step elutions of 30 and 40 mM (lanes 6–9) contained the majority of the TFIIA shift activity. The putative TFIIA-dependent complex observed with these fractions had a slightly reduced mobility relative to that of the complex formed with the control DEAE-5PW TFIIA (Fig. 6A, cf. lanes 9 and 10). This difference is likely attributable to the dilute protein concentration in these fractions, as subsequent experiments showed that titration of BSA in purified TFIIA-containing fractions can modulate the mobility of the TFIIA–TBP complex (data not shown). In any event, the identity of this complex was confirmed by use of immune serum to supershift the complex (data not shown). These observations provide an obvious explanation for our previous failure to affinity-purify TFIIA using Ni-resin loaded with His-tagged yTBP. Another TBP-dependent mobility shift activity, which migrates more rapidly, was also seen in the input and flowthrough fractions (lanes 1,3), but its identity is unknown.

Figure 6. Purification of hTFIIA by affinity chromatography on Ni-resin by use of its intrinsic histidine tag. (A) Mobility shift analysis of fractions obtained by fractionating HeLa nuclear extract on Ni-resin. The mobility shift assay is described in Fig. 5A. [Lane 1 (+yTBP)] 1 μ l of the HeLa nuclear extract (Input); [lanes 2 (–yTBP) and 3 (+yTBP)] 1 μ l of the flowthrough fraction; [lanes 4 (–yTBP) and 5 (+yTBP)] 3 μ l of the 20 mM imidazole (Im) step; [lanes 6 (–yTBP) and 7 (+yTBP)] 3 μ l of the 30 mM imidazole step; [lanes 8 (–yTBP) and 9 (+yTBP)] 3 μ l of the 40 mM imidazole step; [lane 10 (+yTBP)] 0.5 μ l of the DEAE-5PW TFIIA fraction (control). (B) Silver-stained SDS-PAGE assay of selected fractions from the Ni-resin chromatography. (Lane 1) Nuclear extract input (5 μ l); (lane 2) flowthrough (5 μ l); (lane 3) 20 mM imidazole step (5 μ l); (lane 4) 40 mM imidazole step (15 μ l). The positions of the TFIIA subunits are indicated at right. (C) Western analysis shows that Ni-resin chromatographic fractions contain p35. The fractions were probed with α 1-376 antiserum. (Lane 1) Four microliters of HeLa nuclear extract (Input); (lane 2) 4 μ l of flowthrough (F.T.); (lane 3) 4 μ l of the 20 mM imidazole (Im) step; (lane 4) 10 μ l of the 30 mM imidazole step; (lane 5) 10 μ l of the 40 mM imidazole step; (lane 6) 10 μ l of the 100 mM imidazole step. Bands in the 50- to 60-kD range are also seen with preimmune serum. Molecular mass markers in kilodaltons are indicated at left, as well as the 35-kD TFIIA subunit. Similar analyses in Fig. 5B show the presence of p19.

SDS-PAGE analysis of the Ni-resin chromatographic fractions is shown in Figure 6B and visually demonstrates the selectivity of this purification (>400-fold). Subsequent chromatography of the 40-mM imidazole step on DEAE-5PW HPLC (data not shown) indicates that the large TFIIA subunit actually migrates just below the dark band in the region of 35 kD (lane 4). The 12-kD subunit is visible by silver staining, but the weakly staining 19-kD band could only be visualized by Western blot. Immunological analysis of the above fractions clearly shows the retention of the p35 subunit (Fig. 6C, lanes 4,5) of TFIIA, as well as p19 (see Fig. 5B). Similar results were observed when NAM cell nuclear extracts were processed over Ni-resin (data not shown), an important result because two of the cDNA libraries screened were from this cell line. The band between 50 and 60 kD, which elutes at 20 mM imidazole (Fig. 6C, lanes 1,3) is nonspecific, because it is seen by use of both preimmune serum and antigen-blocked immune serum (data not shown). We have not yet detected any specific hTFIIA/ α -encoded 55-kD protein band by these analyses (see Discussion). Moreover, by directly denaturing HeLa cells in 6 M guanidine, followed by Ni-resin chromatography, we could detect p35, but not p55 (J. DeJong

and R.G. Roeder, unpubl.), possibly implying a rapid conversion of p55 to p35 and p19 *in vivo*. A comparison of the silver staining and Western blotting results from both nuclear extracts and purified fractions indicates that TFIIA is a relatively rare species in the cell.

Transcription is reduced in TFIIA-depleted nuclear extracts

Because a requirement for, or even an effect of, TFIIA in transcription has not been consistent, we wished to determine the effect of TFIIA immunodepletion. Moreover, because *in vitro*-reconstituted transcription systems may be permissive as a result of artificially high concentrations of particular components, or the loss of natural constraints, the demonstration of an effect of TFIIA on transcription from nuclear extracts could be physiologically more relevant. To demonstrate the efficacy of the TFIIA depletion, mobility shift analysis was performed with extracts depleted with both preimmune and immune serum (Fig. 7A, lanes 2,3). The TFIIA-TBP shift was clearly missing in the immune serum-treated extracts, whereas other nonspecific shifted complexes remained at similar levels. Transcriptional analysis

A

B

C

Figure 7. Transcriptional analysis of extracts depleted of TFIIA. (A) Mobility shift analysis demonstrates that TFIIA is present in preimmune, but not immune-depleted nuclear extracts. (Lane 1) One microliter of Ni-resin-purified TFIIA control; (lane 2) 1 μ l of preimmune serum (PI)-depleted extract; (lane 3) 1 μ l of immune serum (I)-depleted extract. The position of the shift is indicated at right (D/A). (B) Antiserum against hTFIIA/ α , but not preimmune serum, greatly reduces transcription from the AdML promoter. Nuclear extracts (7 μ l) that were not treated (lane 1), treated with protein A-Sepharose resin alone (lane 2), treated with protein A-Sepharose resin coupled to preimmune serum (lane 3), or treated with protein A-Sepharose resin coupled to immune serum (lane 4) were used to assay transcription. The position of the G-free cassette RNA transcript derived from the pMLC₂AT template is indicated. (C) Two separate sources of TFIIA can restore transcription from TFIIA-depleted nuclear extracts. Extracts were depleted with either preimmune (PI) or immune (I) serum, as shown. Immune serum-depleted reactions additionally contained the indicated amounts of Ni-resin-purified TFIIA or DEAE-5PW fraction TFIIA. The G-free RNA transcript from the AdML promoter is shown (pMLC₂AT).

showed an ~10-fold reduction with the AdML promoter (pMLC₂AT; Sawadogo and Roeder 1985b) in immune versus preimmune serum-treated extracts (Fig. 7B, lanes 3,4). To establish further that the antibody-mediated inhibition was attributable to specific TFIIA–antibody interactions and not simply to nonspecific trapping of transcription components, we added back highly purified DEAE-5PW TFIIA. As shown in lanes 6–8 of Figure 7C, 1 μ l of this fraction restored transcription to nearly the original (preimmune serum) level. To rule out the possibility that nonspecific salt or protein concentration effects caused this restoration, we also added Ni-resin-purified TFIIA. Increasing amounts of this fraction could also restore transcription to nearly original levels (Fig. 7C, lanes 1–5). Because the background population of proteins in these two highly purified samples is expected to be different, we infer that the effects seen were attributable to the common component TFIIA. Transcription assays conducted with a major late template that lacked the upstream stimulatory factor (USF) (Sawadogo and Roeder 1985a; Gregor et al. 1990) site showed essentially the same results (data not shown), suggesting that TFIIA affects the basal transcription machinery acting through the core promoter. Interestingly, depletion experiments consistently showed a residual level of transcription (Fig. 7A, lane 4) which could be attributable to incomplete TFIIA depletion, the presence of TFIIA-substituting activities (Roy et al. 1991), or a residual (intrinsic) ability of the basal transcription machinery to function, albeit weakly, without TFIIA.

Discussion

TFIIA purification and cloning of hTFIIA/ α

Purification of TFIIA was accomplished by use of previously published separation methods (Matsui et al. 1980; Samuels and Sharp 1986; Reinberg et al. 1987), followed by two HPLC chromatographic steps and a yTBP protein affinity column. While this work was in progress several laboratories reported similar affinity purifications (Usuda et al. 1991; Cortes et al. 1992), but the subunit composition of hTFIIA purified from different laboratories is quite different. Calf thymus TFIIA consisted mainly of 19- and 12-kD polypeptides (Samuels and Sharp 1986), whereas HeLa nuclear extract-derived TFIIA was reported to contain either a single 35-kD polypeptide (Usuda et al. 1991; Waldschmidt and Seifart 1992) or 35-, 19-, and 12-kD polypeptides (Cortes et al. 1992; Coulombe et al. 1992). Our present results support the conclusion that TFIIA from HeLa cells exists as a three-subunit complex. The reason for these differences could be the extremely weak silver staining properties of the p19 band and the need to use high percentage polyacrylamide gels to retain the p12 band during electrophoresis. There still exists the possibility that the different sources of material or purification regimens used previously do reveal separate, partly dissociated (e.g., p19 and p12 together) or proteolyzed species of TFIIA.

By use of a synthetic oligonucleotide designed from

two microsequenced peptides, we isolated five independent cDNAs from three separate libraries, all of which were consistent with a derivation from a single mRNA species. Striking homology to the yeast gene TOA1 is evident in regions I and IV of the hTFIIA/ α cDNA, whereas region III shows conservation of net negative charge. It is probable that one or more of these conserved regions could be responsible for the interaction with the highly conserved yTBP and hTBP proteins. This interaction has been partially mapped to the basic region of TBP (Buratowski and Zhou 1992; Lee et al. 1992), suggesting that acidic region III might be involved in charge–charge interactions with TBP and/or with its own histidine residue cluster (residues 81–87). Interestingly, two other proteins, Dr1 and CCG1(TAF250), known to interact with TBP also contain short regions of acidic residues (Inostroza et al. 1992; Takada et al. 1992; Hisatake et al. 1993; Kokubo et al. 1993). It is conceivable that highly acidic regions may be common to at least a subset of TBP-interacting proteins, possibly including acidic activators (Stringer et al. 1990).

It is also likely that one or more of these regions (I, III, and IV) interacts with other conserved transcription factors as well. This idea is supported by evidence that recombinant (heterologous) yTFIIA substitutes for hTFIIA in a reconstituted TFIIA-dependent mammalian transcription system, though it was not as active as purified hTFIIA (Ranish et al. 1992). The divergent region II might be responsible, in part, for any species-specific interactions. This region has a high glutamine content, reminiscent of glutamine-rich activators (for review, see Mitchell and Tjian 1989), although it lacks precise homology with them. Recently, it has been shown that glutamine-rich regions (but not necessarily the glutamine residues per se) are necessary for the interaction of the *Drosophila* coactivator TAF110 with Sp1 (Hoey et al. 1993), although the involvement of hTFIIA/ α region II in specific protein–protein interactions remains to be determined. The hTFIIA/ α -encoded protein also has a potential activation domain in residues 282–298 of the highly acidic region III (for review, see Sigler 1988). In addition, the alternating bulky hydrophobic and flanking acidic residues in region I (residues 17–45) appear somewhat similar to residues critical for VP16 activation (Cress and Treizzenberg 1991). Robson–Garnier secondary structure analysis predicted these amino acids to be a β -sheet structure (data not shown), an interesting result because of recent evidence for the involvement of such a structural motif, rather than acidic character per se, in the function of acidic activators (Leuther et al. 1993). The 7-residue histidine tract is of unknown physiological significance, but such a feature has also been observed in other proteins such as the transcription factor YY1, which also contains an acidic stretch (Shi et al. 1991).

Expression of the hTFIIA/ α clone in *E. coli* and in a coupled transcription–translation system demonstrated that the recombinant protein migrates anomalously (55 kD) compared with both the predicted molecular mass (41.5 kD) and the apparent sizes of the purified TFIIA

subunits. Analysis of carboxy-terminal deletion mutations demonstrated that the difference in predicted and actual migration is attributable, in part, to sequences at the carboxyl terminus, possibly the charged region III. Interestingly, the homologous yeast TOA1 gene predicts a 32-kD- protein, which actually migrates at 43 kD, both when purified and when expressed as a recombinant protein (Ranish and Hahn 1991; Ranish et al. 1992).

The presence of an intrinsic affinity tag in the deduced p55 sequence suggested a simple TFIIA purification scheme. The interaction of TFIIA with Ni-resin was demonstrated by mobility shift analysis of imidazole-eluted fractions, and the identity of the shifted complex was confirmed by supershifting with immune antisera. This finding should accelerate studies of TFIIA structure and could also provide a rapid and clean source of this factor for use in functional studies. This is important from a practical standpoint, because clones for the other hTFIIA subunit have not yet been reported.

TFIIA structure

The relationship between hTFIIA/ α and the p35 and p19 subunits of purified TFIIA is, except for the differences in mobility, almost identical, as indicated by a number of observations. First, identity was observed between peptides from both the p35 and p19 subunits and the cDNA deduced sequence (48 residues). Second, antibodies raised against the recombinant protein displayed immunological specificity for TFIIA, reacting with both the 35- and 19-kD subunits. Thus, it is not coincidental that the sum of the sizes of the two large TFIIA subunits is ~55 kD. Third, the presence of an intrinsic histidine tag in natural TFIIA was predicted from the deduced hTFIIA/ α sequence and was confirmed biochemically. Fourth, the recombinant protein was shown to be functional, because it could substitute for the p35 and p19 subunits in a TBP-dependent mobility shift assay.

These data all support the idea that hTFIIA/ α encodes a protein that is co- or post-translationally processed (or degraded during cell disruption) to form the p35 and p19 subunits of natural TFIIA. Our inability to detect the p55 protein in either cell extracts or purified fractions suggests that this process is quite rapid. The simplest and most likely scenario would involve a single cleavage of p55 toward the carboxyl terminus to produce an amino-terminal p35 subunit and a carboxy-terminal p19 subunit. Our preliminary observations of p35 reactivity with amino- and carboxy-terminal-specific antisera (data not shown), as well as the short amino-terminal P1 peptide that is apparently derived from p19, however, raise the less likely possibility that p19 might be produced by a fusion of amino- and carboxy-terminal p55 sequences (Shub and Goodrich-Blair 1992). In any event, a complete understanding of the necessity for, and mechanism of, events leading from p55 to p35/p19 will require the expression of hTFIIA/ α in vivo and the use of better region-specific antibodies.

Although in all likelihood hTFIIA/ α encodes the p35 and p19 subunits, it is hypothetically possible that p55 is

a subunit of a heretofore undiscovered TFIIA-like protein and that p35 and p19 are encoded either by alternatively spliced p55 mRNA species or by highly homologous genes. Screening results from three libraries, however, gave no evidence for related cDNA species, an observation substantiated by PCR analysis of reverse-transcribed HeLa poly(A) mRNA with p55-specific primers (J. DeJong and R.G. Roeder, unpubl.). Interestingly, although Northern blotting analysis with a hTFIIA/ α probe revealed two unexpectedly large bands of 6.5 and 7.0 kb, other hybridization experiments demonstrated that both of these mRNA species contained region II (J. DeJong and R.G. Roeder, unpubl.), a result inconsistent with alternative splicing. Thus, although the existence of multiple TFIIA-like proteins cannot be excluded with complete certitude, it appears likely that two genes (hTFIIA/ α and hTFIIA/ β) will be sufficient to encode human TFIIA and that they correspond to the yeast genes TOA1 and TOA2 (Ranish et al. 1992). This idea is supported by preliminary studies with TFIIA reconstituted entirely from recombinant components (R. Bernstein, J. DeJong, and R.G. Roeder, unpubl.).

Functions of TFIIA

Reconstitution of recombinant hTFIIA activity, as well as structure-function analysis, will require the isolation of the human counterpart of the yeast gene TOA2, or substitution with heterologous subunits from other species. As these were unavailable, we made polyclonal antiserum to hTFIIA/ α for use as a probe of TFIIA function, and to study the relationship between hTFIIA/ α and natural TFIIA. The transcription assays presented here clearly demonstrate an effect of TFIIA on transcription in vitro. Moreover, the dramatic reduction in transcription on TFIIA depletion was reversible by the addition of TFIIA that was highly purified by either of two independent methods. In support of this result, reduced transcription in extracts of terminally differentiated avian erythroid cells was interpreted to be attributable to limiting levels of TFIIA, because readdition of this factor restored transcription (Bungert et al. 1992).

It has been proposed that TFIIA functions by stabilizing the assembly of general transcription factors in a transcriptionally competent preinitiation complex. Initially, this idea was supported by the results of mobility shift assays, which showed an apparent TFIIA requirement for entry of TFIIB and the remaining factors (Buratowski et al. 1989). This result, however, is highly dependent on the mobility shift conditions, and recent experiments demonstrate that TFIIA can interact with TBP at any stage during the assembly of the preinitiation complex (Maldonado et al. 1990; Cortes et al. 1992). Moreover, numerous proteins with apparently different roles in transcription can stabilize the TBP-DNA mobility shift complex, including NC1, NC2, and Dr1. Thus, it is possible that TFIIA stimulates transcription through a mechanistic step that follows TBP binding and that may involve a TBP conformational change (Lee et al. 1992). Interestingly, because RNA polymerases I, II, and

III are known to require TBP (for review, see Sharp 1992), TFIIA might affect each similarly, an idea supported by evidence for TFIIA involvement in the RNA polymerase III-transcribed U6 gene (Waldschmidt and Seifart 1992).

A surprising number of proteins, including TAFs (Pugh and Tjian 1990; Dynlacht et al. 1991; Takada et al. 1992; Zhou et al. 1992) and the carboxy-terminal domain (CTD) tail of the large subunit of RNA polymerase II (Koleske et al. 1992; Usheva et al. 1992) have been demonstrated recently to interact with TBP. Upstream factor-TBP interactions have also been documented for VP16 (Stringer et al. 1990), E1A (Lee et al. 1991), Zta (Lieberman and Berk 1991), PU.1 (Hagemeier et al. 1993), and p53 (Truant et al. 1993). Determining whether TFIIA and these factors interact simultaneously with TBP and whether these interactions are physiologically important will be a major challenge. Interestingly, α TFIIA antiserum-dependent supershifts of immunologically selected TFIID complexes suggest that TFIIA and TFIID (TBP) are associated in the cell (C.-M. Chiang, J. DeJong, and R.G. Roeder, unpubl.). It was shown previously that preincubation of TFIIA and general factors with the promoter caused a modest decrease in the ability of Sp1 and USF to activate transcription in a reconstituted system containing the factor USA, even though TFIIA was otherwise required for high-level induction (Meisterernst et al. 1991). Additionally, the Gal4-AH-directed transition from a closed to an open promoter complex was reported to be rate limited by a Gal4-AH, TFIIA, and TFIID association step, implying a direct positive involvement of TFIIA in transcription activation (Wang et al. 1992).

TFIIA may interfere with the formation of nonfunctional TBP (TFIID)-negative factor complexes (Meisterernst et al. 1991; Meisterernst and Roeder 1991; Inostroza et al. 1992). Mobility shift assays with a variety of proteins, including Dr1, NC1, and NC2 have demonstrated a competition *in vitro* for binding to TBP. Transcription in one system showed that the TFIIA requirement is dependent on TFIID as the source of TBP and was thought to reflect the displacement of negative factors (Cortes et al. 1992). It is likely that proteins interacting with TBP at common sites, possibly including the large subunit in TFIID (Takada et al. 1992; Hisatake et al. 1993) would have some degree of competition with TFIIA for TBP binding. The negative effects of the large TFIID subunit on TBP binding (Kokubo et al. 1993) suggest the possibility that TFIIA may influence, alone or in combination with activators, the promoter-binding properties of TFIID itself. If so, TFIIA will have a key role in regulating the formation of competent transcription complexes because, in contrast to other TBP-binding cofactors, it facilitates the initiation of transcription. Potentially consistent with this is the observation that TFIIA is present in the cell in relatively low amounts. The related possibility of activator-TFIID (TBP) interactions mediating a similar role during activation may also deserve investigation. In any event, differing levels of negative factors could provide an explanation for the variable TFIIA requirement from different laboratories, in addition to the possibility of TFIIA cross-contamina-

tion (in various chromatographic fractions) and the existence of TFIIA-substituting activities (for review, see Roeder 1991).

An alternative hypothesis for TFIIA function, based on the potential similarity of hTFIIA/ α to acidic and glutamine-rich classes of transcriptional activators (for review, see Sigler 1988; Mitchell and Tjian 1989), is that TFIIA, by associating with TBP (or TFIID), might itself activate transcription. Our isolation of a hTFIIA subunit cDNA is an important first step in investigating this intriguing possibility, as well as other aspects of TFIIA function.

Materials and methods

Purification of TFIIA

HeLa cell cytoplasmic supernatants (100,000g, S-100), prepared as described (Dignam et al. 1983), were dialyzed against buffer A [20 mM Tris-HCl (pH 7.9), 20% glycerol, 0.2 mM EDTA, 0.125% 2-mercaptoethanol, and 0.5 mM phenylmethylsulfonyl-fluoride (PMSF)] containing 100 mM KCl. S-100 was spun at 35,000 rpm for 90 min in a Ti45 rotor to remove lipids and other insoluble material. The clarified S-100 (1.1 liters, 15 grams of protein) was divided into two batches and loaded onto separate 400-ml phosphocellulose (P11; Whatman) columns and washed with buffer B [20 mM Tris-HCl (pH 7.9), 20% glycerol, 0.05% NP-40, 2.5 mM dithiothreitol (DTT), and 0.5 mM PMSF] containing 100 mM KCl. The P11 flowthrough fractions were loaded directly onto 175-ml Q-Sepharose FF (Pharmacia) columns equilibrated with buffer B. Material eluting in the 0.2–0.5 M KCl step was dialyzed against buffer C [30 mM HEPES (pH 7.9), 20% glycerol, 300 mM KCl, 1 mM DTT, 0.05% NP-40, and 0.5 mM PMSF] containing 100 mM KPi. The dialyzed material was divided into batches and loaded onto 20-ml hydroxylapatite (Bio-Gel HT; Bio-Rad) columns. Bound TFIIA activity was eluted in buffer C containing 350 mM KPi. The eluted material from each HAP column run was pooled and dialyzed against buffer D [30 mM HEPES (pH 7.9), 10% glycerol, 300 mM KCl, 1 mM DTT, and 0.5 mM PMSF] containing 100 mM KPi. The dialyzed material was loaded onto a Bio-Gel HPHT HPLC hydroxylapatite column (Bio-Rad) and eluted with a linear gradient from 100 to 300 mM KPi. Eluted material (200–250 mM KPi) from each of three runs was pooled separately as early eluting upper mobility shift (U-IIA) and later eluting lower mobility shift (L-IIA) complexes. The activities were dialyzed against buffer E [20 mM HEPES (pH 7.9), 10% glycerol, 0.2 mM EDTA, and 0.5 mM PMSF] containing 100 mM KCl. These dialysates were loaded in batches onto a Bio-Gel DEAE-5PW HPLC column (Bio-Rad), and TFIIA was eluted with a linear gradient from 100 to 500 mM KCl. Active fractions (~250 mM KCl), which now contained a predominance of the L-IIA activity, were pooled and dialyzed against buffer F [20 mM HEPES (pH 7.9), 10% glycerol, 0.2 mM EDTA, 0.05% NP40/1 mM DTT, and 0.5 mM PMSF] containing 125 mM KCl. Histidine-tagged bacterially expressed recombinant γ TBP, prepared as described (Hoffmann and Roeder 1992), was coupled to Affigel-10 resin (Bio-Rad) to a final concentration of ~1.5 mg of TBP/ml of resin according to the manufacturer's instructions. The TFIIA DEAE-5PW input material was loaded onto multiple 0.2- to 0.5-ml γ TBP-affinity columns, and the flowthrough was recycled over the column several times. After extensive washing with buffer E containing 150 mM and 175 mM KCl, TFIIA was eluted with 0.7 M KCl. The affinity column eluates were combined, adjusted to final concentrations of 1% NP-40 and 12.5% TCA to precipitate TFIIA,

washed with acetone, and resuspended in buffer B containing 100 mM KCl. The final preparation was estimated to contain ~5 µg of each of the 35-, 19-, and 12-kD bands. Following SDS-PAGE [Laemmli 1970], the TFIIA subunits were transferred to PVDF membranes (Immobilon, Millipore) and stained with Ponceau S and/or Amido black (Sigma). Strips containing the stained bands were excised and given to the Rockefeller University Protein Sequencing Facility for processing. After protease digestion in situ with endoproteinase LysC in the case of p19, or trypsin in the case of p35, the peptides were eluted and resolved on a microbore reverse-phase HPLC column and sequenced [Fernandez et al. 1992].

Purification of TFIIA from HeLa, as well as NAM, nuclear extracts by Ni-resin affinity chromatography [Hochuli et al. 1987] was done by passing the extract (20 ml, 140 mg) onto a 2-ml Ni-resin (Novagen) column equilibrated with buffer B containing 200 mM KCl. Fractions were eluted in the same buffer containing increasing imidazole concentrations (20, 30, 40, and 100 mM). Bovine serum albumin (BSA) was added to a final concentration of 100 µg/ml to the 30, 40, and 100 mM imidazole steps to help stabilize the dilute protein in these fractions, which were then dialyzed against buffer B containing 100 mM KCl to remove the imidazole. To obtain TFIIA for sequence analysis as described above, nuclear extracts (500 ml) were processed over phosphocellulose P11, Ni-resin, and DEAE-5PW HPLC, in that order.

Cloning and sequencing of hTFIIA/α

Two of four peptides (P3, P4) obtained from the Rockefeller University Sequence Facility could be contiguously aligned to the carboxyl terminus of the yeast TFIIA gene TOA1, and these peptides created the combined sequence GIMNLNGRDYIFSK-IGDAEW. The gap was filled using a hypothetical alanine residue based on the TOA1 sequence. A set of fourfold degenerate 63-mer oligonucleotides, which also contained the neutral base inosine (I), was designed (5'-GGIATATGAACCTIAATGGIC/AGIGATTACATITTCA/TCCAAIGCIATIGGIGATGCI-GAITGG-3'). This probe was kinase labeled with [γ -³²P]ATP (ICN) with T4 polynucleotide kinase (Boehringer Mannheim) and used to screen the two NAM cell cDNA libraries (kindly provided by Dr. Craig Rosen, Roche Institute of Molecular Biology, Nutley, NJ; Scheidereit et al. 1988). Oligonucleotide screening was performed in 5× Denhardt's solution, 1% SDS, 6× SSPE, 100 µg/ml of denatured salmon sperm DNA. First-round filters were hybridized overnight at 42°C and washed with 6× SSPE, whereas the temperature of subsequent rounds was raised to 58°C to eliminate nonspecific hybridization. Screening of a random-primed BJA-B cDNA library (Stratagene) with oligo-labeled cDNA inserts was done by established techniques. Final positive phage signals were excised in vivo into pBluescript II SK (Stratagene) according to the manufacturer's instructions and identified by the dideoxy sequencing method [Sanger et al. 1977] with Sequenase (U.S. Biochemical). The nucleotide sequence of the open reading frame was determined on both strands by sequencing subclones and synthesizing internal sequencing primers.

Expression of hTFIIA/α in *E. coli*

The initial λ17-1 clone ended at methionine position 40, so the convenient *Nde*I site at position 129 was used to subclone a *Nde*I-*Eco*RI fragment into pET11d (Novagen) to allow expression of amino acids 129–376 with an amino-terminal His tag (pET11d129-376), as well as without a His tag (pET11a129-376). Subsequently a full-length clone (λ11) was isolated and an

amino-terminal PCR primer (5'-GGGCATATGGCGAACTCG-GCAAA-3') was synthesized to encompass the initiating methionine and to create an *Nde*I site. Using a downstream opposite strand primer (5'-GAACAGGAGTCACACCTGC-3') [residues 147–154], a 450-bp PCR fragment was produced and subcloned into the *Sma*I site of pGEM (Promega) and sequenced. The internal *Nde*I fragment was subcloned into the pET11a129-376 construct described above to create pET11aN1-376. The *Nde*I fragment was also subcloned into the *Nde*I site of pRSETB (Invitrogen) to encode residues 1–129 with a His tag at the carboxyl terminus (pRSET-A/α1-129). An *Alu*I (nucleotide 1065)-*Eco*RI fragment expressing 73 carboxy-terminal amino acids was subcloned into a *Pvu*II-*Eco*RI site in pRSETB to allow expression of this polypeptide with an amino-terminal His tag (pRSET-A/α304-376).

All constructs were grown in the BL21(DE3)pLysS *E. coli* strain (Novagen) at 37°C in LB media plus 150 µg/ml of ampicillin. Cells were induced by the addition of 2 mM IPTG when the cells were between 0.1 and 0.3 OD₆₀₀. Cells were generally harvested 3 hr postinduction and solubilized in 6 M guanidine, 100 mM NaH₂PO₄, 200 mM KCl, 2 mM β-mercaptoethanol, and 5% glycerol, overnight at –20°C. The solubilized material was sonicated five times for 30 sec each and spun at 8000 rpm for 15 min. The supernatant was incubated at 4°C for 1 hr with Ni-resin (0.75 ml/liter culture). The resin was processed batchwise with urea substituting for guanidine at pH 8, pH 6.3, pH 5.9, and pH 4.5 as described by Invitrogen.

Antibody production

Polyclonal antiserum was raised against full-length hTFIIA/α protein as well as against specific regions (see Fig. 4A). Full-length and residue 129–376 polypeptides were prepared by resolution of Ni-resin-purified material on SDS-PAGE, staining with cold 0.25 M KCl, and elution of the excised gel fragments (Elutrap, Schleicher & Schuell). Amino-terminal and carboxy-terminal polypeptides were sufficiently pure to inject directly following Ni-resin purification. After obtaining preimmune serum, ~500 µg of recombinant protein was mixed 1 : 1 with complete Freund's adjuvant and injected subcutaneously into New Zealand white rabbits by the Rockefeller University Laboratory Animal Research Center. After 4 weeks the rabbits were boosted with ~100–200 µg of protein mixed 1 : 1 with incomplete Freund's adjuvant. First-boost immune sera were collected 10 days later.

Transcription

To prepare nuclear extracts for transcription, protein A-Sepharose (Pharmacia) was first coupled to serum as follows. Swollen protein A-Sepharose (600 µl) was mixed with 600 µl of crude preimmune or immune serum raised against residues 129–376. Binding of IgG was done for 90 min at 4°C followed by two washes with 3 ml 0.2 M Na-borate (pH 9.0). Diaminodipropylamine (DMP, 20 mg) was added to each tube and incubated at room temperature for 30 min. The cross-linked resin was spun and resuspended in 0.2 M ethanolamine (pH 8.0) to block any unreacted DMP. The efficiency and amount of IgG cross-linked was ascertained by SDS-PAGE of boiled samples taken both before and after cross-linking, and was ~1 mg/ml. These resins were incubated with 1 mg/ml of BSA for 1 hr at 4°C to block nonspecific binding sites. Control protein A-Sepharose resin was not cross-linked but was incubated with 1 mg/ml of BSA.

To see the effects of depletion by the antibody-coupled resins, 500 µl of HeLa nuclear extract was incubated with 250 µl of immune resin, preimmune resin, control resin, or no resin. The

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salt concentration was adjusted to at least 300 mM NaCl, and the depletion was allowed to proceed for 3 hr at 4°C, followed by a 3-hr dialysis against buffer B.

Transcription assays (Sawadogo and Roeder 1985b) were performed in 40 mM HEPES (pH 8.4), 7 mM MgCl₂, 500 μM ATP and UTP, 10 μM CTP, 100 μM *O*-methyl GTP, 1.6 units of RNasin (Promega), 5 mM DTT, and 5% glycerol. Template DNA (pMLC₂AT) was purified on a cesium chloride gradient and digested with *Sma*I to linearize the template at the end of the G-free cassette. Assays (20–50 μl final volume) were performed by adding buffers, nuclear extract (8 μl), template (200 ng), and TFIIA fractions together for a 15-min preincubation at 30°C. The transcription reaction was initiated by the addition of nucleotides and [³²P]CTP (ICN), and allowed to continue at 30°C for 1 hr. The reaction was stopped with 8 M urea, 10 mM Tris-HCl (pH 7.5), 10 mM EDTA, 0.5% SDS, 100 mM LiCl, 200 μg of tRNA, and 300 mM Na-acetate and extracted with an equal volume of phenol/chloroform (1 : 1). The ethanol-precipitated transcripts were dissolved in formamide loading dye for electrophoresis on 8 M urea/TBE gels.

Other procedures

Mobility shift assays (Fried and Crothers 1981) were performed by use of a TATA box-containing oligonucleotide derived from –45 to –15 of the AdML promoter. Approximately 10 fmoles of probe was incubated with 50 fmoles of yTBP in the presence or absence of various TFIIA fractions at 37°C for 30 min. Reactions were performed in 10 mM HEPES (pH 7.9), 2% (wt/vol) PEG-8000, 60 mM KCl, 5 mM DTT, 0.2 mM EDTA, 4 mM MgCl₂, 5 mM ammonium sulfate, and 8% glycerol (Maldonado et al. 1990) containing 100–1000 ng of poly[d(I-C)] (Pharmacia) as competitor. Reactions were loaded onto 5% acrylamide (50 : 1 acrylamide/bis), 5% glycerol TBE gels for separation of bound complexes and free probe. Coupled *in vitro* transcription–translation (TnT, Promega) was performed with 2 μg of linearized template (pBluescript II SK containing the insert excised from the λ11 phage) in the presence of [³⁵S]methionine (ICN) as described by the manufacturer. Labeled polypeptides were separated by 15% SDS-PAGE and exposed to X-ray film after drying. Western blotting with α129–376 and α1–376 antisera (1 : 7500 dilution) was performed using the ECL system (Amersham) according to the manufacturer's instructions. Elution and renaturation of SDS-PAGE-separated TFIIA subunits was done using PVDF membrane essentially as described by Szewczyk and Summers (1988). Computer analysis was done by use of MacVector 4.0 (IBI), and data base searches were done by use of the program of Pearson and Lipman (1988).

Note added in proof

Additional peptide sequences obtained from the p35 subunit (LXENKLMQS) and the p19 subunit (AEDGQVEPL/pN/INXDD), which span amino acids 45–53 and 304–319, respectively, have recently provided further support for our conclusions. The amino acid sequence data reported in this paper have been submitted to the EMBL data library.

Acknowledgments

We thank Drs. Giorgio Lagna, Dong Kun Lee, Eric Sinn, and Jong Bok Yoon, as well as Richard Bernstein, for valuable technical advice and materials and/or for critical comments on the manuscript. We thank Joe Fernandez and Sheena Mische for performing amino acid sequence analysis at the Protein Se-

quence Facility of The Rockefeller University, which also provided synthetic oligodeoxynucleotides. J.D. is supported by an American Cancer Society Fellowship PF-3650. This work was also supported by National Institutes of Health grants CA42567 and AI27379 to R.G.R. and by general support from the Pew Trusts to the Rockefeller University.

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Genes Dev. 1993, **7**:

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