TFII-I is a multifunctional phosphoprotein with roles in transcription and signal transduction. Here we report characterization of three additional alternatively spliced isoforms of TFII-I. Employing isoform-specific antibodies, we show that the isoforms form a stable complex in vivo preferentially in the nucleus compared with the cytoplasm. We further show that both homomorphic and heteromeric interactions are possible and that the heteromeric interactions between a wild type and a nuclear localization-deficient mutant result in nuclear translocation of the complex, leading us to postulate that complex formation might aid in nuclear translocation. In functional assays all four isoforms individually bind to DNA and transactivate reporter genes to a similar extent. However, although co-expression of different TFII-I isoforms leads to enhanced basal activity, it results in attenuated signal responsive activity. Thus, TFII-I might differentially regulate its target genes via complex or subcomplex formation.

TFII-I is an intriguing transcription factor, with broad biochemical and biological activities and may be involved in several genetic disorders (1–8). TFII-I functions through the Inr element both in vitro (1, 4, 9–11) and in vivo (1, 5, 11, 12). It also functions through upstream regulatory elements in the adenovirus major late promoter and in c-fos promoter in vivo (2, 3, 12). Based on its unique physical and functional interactions at both the Inr element and upstream regulatory sites, TFII-I is postulated to be a novel transcriptional cofactor that integrates signals from the regulatory components to the basal machinery (1, 12). We have recently shown that TFII-I is phosphorylated at both serine and tyrosine residues and that tyrosine phosphorylation of TFII-I is critical for its transcriptional properties (5). Equally interesting is the observation that a variety of extracellular signals mediating through cell surface receptors, including growth factor receptors, lead to enhanced tyrosine phosphorylation and increased transcriptional activity of TFII-I (3, 5, 7). TFII-I is a ubiquitous protein partitioned in the cytoplasm and nucleus (7). In the B cell cytoplasm a significant fraction of TFII-I is associated constitutively with Bruton’s tyrosine kinase (7), mutations in which lead to X-linked immune deficiency in humans and mice (14–16). TFII-I is tyrosine-phosphorylated by Bruton’s tyrosine kinase in vitro (13), and upon immunoglobulin receptor cross-linking in B cells it is released from Bruton’s tyrosine kinase to enter the nucleus (7). These observations suggest that TFII-I is downstream of several signal transduction pathways and may be a mediator linking signal-responsive activator complexes to the general transcription machinery, perhaps in a cell type-specific fashion.

Recent genetic data suggest that TFII-I belongs to a family of proteins, each having the I-repeat first identified in the founding member TFII-I (8, 12, 17, 18). Interestingly, both TFII-I and the related protein WBSC11 (18) have been mapped to the breakpoint regions of the 7q11.23 Williams-Beuren syndrome deletion (8, 18). Furthermore, genetic analysis by Francke and co-workers suggest the presence of several alternatively spliced isoforms of TFII-I both in humans and in mice (8). Based on these predicted protein sequences (8), we cloned TFII-I isoforms by using a PCR based strategy (see “Experimental Procedures”) and ectopically expressed and biochemically analyzed them. Here we show that in addition to the 957-amino acid form of TFII-I (Δ), three other alternatively spliced isoforms exist in human, henceforth referred to as α (977 amino acids), β (978 amino acids), and γ (998 amino acids). Compared with the Δ-isoform, the α-isoform contains an additional 20 amino acids (encoded by exon A), the β-isoform contains an additional 21 amino acids (encoded by exon B), and the γ-isoform, which arises by the presence of both exons A and B, contains 41 additional amino acids (8). All four isoforms, when ectopically and individually expressed in COS cells, exhibit similar subcellular distribution. Use of isoform specific antibodies allowed us to demonstrate stable complex formation between the various isoforms either when co-expressed ectopically or present endogenously in eukaryotic cells. The endogenous complex was preferentially located in the nucleus compared with the cytoplasm, and the co-expression of wild type isoform with a nuclear localization-deficient mutant resulted in the localization of the complex to the nucleus. All four isoforms, when expressed in recombinant forms, exhibit similar DNA binding characteristics and bind to both the Inr element of the Vβ promoter and the upstream regulatory site overlapping the serum response element (SRE) of the c-fos promoter (2, 3). However, although the isoforms possess similar homomorphic transactivation potentials, heteromeric complex formation leads to differential activation of reporter genes. Taken together, these data suggest that the complex formation is a means by which the nuclear localization and the transcriptional activity of TFII-I are regulated.
**Isoforms of TFII-I**

**Experimental Procedures**

**Cell Culture—** COS7 cells were grown in Dulbecco's modified Eagle's medium (DMEM: Cellgro) containing 10% fetal bovine serum (Sigma), 50 units of penicillin/ml, and 50 μg of streptomycin (Life Technologies, Inc). Cells were seeded at 37 °C under 5% CO2.

**DNA Constructs—** The isoform having both exon A and exon B (TFII-IAB) and primer 4 (5'-AAACAGCCCCTATCGAAGCCTTTGCAAGGAAGCCACCATTCTTCA-GATGTTGATGAA-GTTTCAGAAGGGCCTGCTTGAATGTTATA-3') and primer 5 (5'-GGGCGGGCGTCGACCACGTGGG-3') (reaction 1). A separate PCR reaction was done by using primer 3 (5'-GAGTGTGGAAGAAACACGCCCCTATCGAAGCCTTTGCAAGGAAGCCACCATTCTTCA-GATGTTGATGAA-GTTTCAGAAGGGCCTGCTTGAATGTTATA-3') and primer 4 (5'-GGGCGGGCGTCGACCACGTGGG-3').

**Isoforms of TFII-I—** To construct the isoforms of TFII-I, the sequences of exon A and exon B (5'-CACGTGGTACAGGTACCACTGTGGA-3') and primer 2 (5'-TGGTACAGGTACCACTGTGGA-3') (reaction 1); primer 3 (5'-CAGAAACAAGTGAGGACCCT-TCCTCACTTGTTTCTGAAGGGACATGTTGA-3') and primer 1 (5'-GTGGATCCACCATGGGCA-9').

**Antibodies—** Antibodies specific to TFII-Iα (and β) isoforms were raised in rabbits (Research Genetics), employing the synthetic peptide (ECL, Amersham Pharma).

**Western Blot Analysis—** Western blot was performed with an anti-TFII-I antibody, pooled, and dialyzed against buffer B (20 mM Tris-Cl, pH 7.9, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenethylsulfonyl fluoride, and 10% glycerol) containing 100 mM NaCl. The lysate was centrifuged at 12,000 rpm for 30 min. The His6-tagged TFII-I isoforms were purified by loading the lysates on to a 1-ml TALON column (CLONTECH) at 4 °C. The column was washed sequentially with 10 column volumes of BC100 media (without detergent and protease inhibitors) and BC100 containing 20 mM imidazole. The tagged protein was eluted with 4 column volumes of BC100 containing 100 mM imidazole. TFII-I isoform—containing peak fractions were analyzed by Western blot with an anti-TFII-I antibody, followed by 12% SDS-PAGE and transferred to Immobilon-P membranes. The membranes were probed with a mouse monoclonal antibody against TFII-I and a polyclonal antibody raised against the synthetic peptide DVDEKQPLSKPLQ (Novagen)

**Electrophoretic Mobility Shift Analysis (EMSA)—** EMSA reactions were performed with Inr or SRE probes in 10 μl of binding buffer containing 50% glycerol, 0.1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 10% glycerol.

**Immunoprecipitation—** For immunoprecipitation, nuclear and cytoplasmic extracts were prepared from Ramos cells according to published procedures. Cells were harvested and resuspended in 400 μl of hypotonic buffer (20 mM Tris (pH 7.9), 5 mM NaF, 2 mM Na3VO4, and 0.5 mM EDTA). The lysate was centrifuged at 10,000 rpm for 30 min.
1 mM Na₃P₂O₇, and protease inhibitor mixture). The resuspended cells were incubated in ice for 15 min and lysed by 5 passages through a 25-gauge needle. The nuclei were collected by centrifugation at 5,000 rpm for 10 min at 4 °C. Nuclear proteins were extracted by resuspending the nuclei in 200 μL of the lysis buffer containing 420 mM NaCl and 25% glycerol and incubating in ice for 30 min. For the immunoprecipitation assay, either 37.5 μg of nuclear or 75 μg of cytoplasmic extract was incubated with anti-TFII-I or with preimmune serum in a final volume of 400 μL of BC100 at 4 °C for 20 min. 10 μL of protein A-Sepharose (50% slurry) was then added and incubated further for 1.5 h at 4 °C, after which the immune complexes were washed 4 times with buffer BC100 containing 0.1% Triton X-100, and 4× SDS sample buffer was added. The beads were heated to 100°C for 3 min, analyzed by SDS-PAGE, and immunoblotted successively with anti-TFII-Ia, anti-TFII-Ig, and anti-TFII-I antibody (4).

GST Pull-down Assay—Whole cell extracts (200 μg) from COS7 cells cotransfected with TFII-I isoforms were subjected to precipitation with glutathione-Sepharose (35 μL, 1:1 slurry; Sigma) for 2 h with rocking at 4 °C. At the end of the incubation the beads were washed 3 times with 1 mL of buffer BC100 containing 0.1% Triton X-100. After the final wash, 4× SDS sample buffer was added to the beads and the beads were heated to 100°C for 3 min, analyzed by SDS-PAGE, and immunoblotted with anti-GFP antibody (CLONTECH). The blots were then stripped to remove the anti-GFP immunocomplex (5) and reprobed with anti-GST antibody.

Transient Transfection and Immunofluorescence—COST cells were transfected with either the GFP construct alone or cotransfected with GFP-tagged TFII-I isoforms as described (1). 30 h post-transfection, cells were fixed with 4% paraformaldehyde and prepared for immunofluorescence. For detection of the splice variants by immunostaining, the protein A-Sepharose-purified TFII-Ia or TFII-Ig antibody were used at a dilution of 1:2500 in conjunction with Alexa™594 goat anti-rabbit IgG (H+L) (Molecular Probes) at a dilution of 1:20,000. Nuclei were stained with 4.6-diamidino-2-phenylindole (DAPI) dye (Sigma). Immunofluorescence was detected using a fluorescence microscope (Nikon, E400).

Reporter Assays—Transient transfection and luciferase reporter assays were done essentially as described (1) with the following modifications. Transfection mixtures were added to cells at 80 to 90% confluence, and after 5 to 6 h of incubation with the transfection mixtures, the cells were trypsinized, and each well of 6-well plates was seeded with 4–5 × 10⁴ cells. Transfection was done with LipofectAMINE (Life Technologies, Inc.). In an Eppendorf tube, either 600 ng of Vβ (3) or c-fos (3) reporter plasmids with or without different TFII-I isoform expression constructs (400 ng when transfected individually and 200 ng each when cotransfected in combinations) and 35 ng of Renilla luciferase plasmid (pRL-TK; Promega Corp.) were mixed. The mixture was then centrifuged at 400 g of empty vector plasmid (pEBG) was also added to normalize the total amount of DNA. The final volumes were adjusted to 100 μL with DMEM. In a separate tube, 6 μL of LipofectAMINE was mixed with 94 μL of DMEM. The plasmid-containing medium was mixed with LipofectAMINE-containing medium and incubated at room temperature for 45 min. At the end of the incubation period cells were washed two times with DMEM, and 500 μL of additional DMEM was added to the cells. The DNA-lipid complex was then added to the cells and incubated overnight in a CO₂ incubator at 37 °C. After 12–14 h of incubation, 1 mL of DMEM containing 20% fetal bovine serum was added to each of the wells, and the incubation was continued for another 8 h. In reporter assays having the Vβ promoter, the media was exchanged at the end of incubation with normal media (DMEM containing 10% fetal bovine serum) and incubated for an additional 12–14 h. In experiments with c-fos reporter plasmid, the transfection media was exchanged with DMEM, and the cells were then serum-starved for 12–14 h. Finally, the cells were stimulated with human EGF (Sigma) at a final concentration of 25 ng/mL for 4 h. The cells were washed twice in PBS and lysed, and the luciferase activities were determined according to manufacturer’s protocol (dual luciferase assay; Promega Corp.).

RESULTS

Analysis of Alternatively Spliced Isoforms of TFII-I by Isoform-specific Antibodies: Preferential Formation in the Nucleus—Both by Western blot analysis and by immunoprecipitation assays using an anti-TFII-I antibody, TFII-I protein always appeared as a doublet (120 and 128 kDa) in either human or murine cells (4, 6, 7). Limit digestion and microsequencing of the biochemically isolated 128-kDa form from HeLa cells yielded two peptide sequences that matched completely with the published TFII-I sequences (data not shown), suggesting that the 128-kDa isoform arises either by post-translational modifications of the 120-kDa form or by alternative splicing. However, because the genomic analysis suggested that there exist three additional isoforms of TFII-I that would arise by alternative splicing (8), we pursued this latter idea. To test the existence of these putative isoforms and to biochemically characterize them further, we employed a PCR-based cloning strategy starting with the published sequence of TFII-I as the template (12). We successfully cloned the three additional isoforms of TFII-I and named them α (977 amino acids), β (978 amino acids), and γ (998 amino acids) (also see Ref. 8). The shortest form (957 amino acids) henceforth in this paper will be referred to as the Δ-isoform. The schematic diagram of these isoforms is shown in Fig. 1A.

Antipeptide polyclonal antibodies were prepared against the peptide α and peptide β (“Experimental Procedures”). Note that because both α and β sequences are present in the γ-isoform, no unique sequence was present in this isoform that could be used to raise an antibody. Hence all the antibodies recognized this isoform. The alternatively spliced isoforms were expressed in COS cells to permit their biochemical characterization and to test the specificity of the antibodies. All four of the isoforms were subcloned into pEBG vector (1, 19) and expressed in COS cells as GST-hexahistidine-tagged fusion proteins. The expression of each protein was tested after purification over the TALON column by Western blot analysis using an anti-TFII-I peptide polyclonal antibody that recognizes all four isoforms (Fig. 1B, left panel). Having ensured that the expression of these proteins was similar and the loading was equivalent, we stripped the blot of immune complexes and reprobed with the anti-α peptide antibody (Fig. 1B, middle panel). In parallel, an identical blot was probed with an anti-β peptide antibody (Fig. 1B, right panel). As expected, although anti-α antibody recognized the α- and the γ-isoforms but not the Δ- and β-isoforms, the anti-β antibody recognized the β- and the γ-isoforms but not the Δ- and α-isoforms, demonstrating isoform/exon specificity of these antibodies. Subsequently, endogenous existence of the α- and β-isoforms was confirmed by the anti-β specific antibodies (data not shown).

Because all the isoforms were preferentially found in the nucleus, we wished to test whether they translocate independently or as heteromeric complexes. We further rationalized that if they interact to form a complex then we should be able to immunoprecipitate the complex with any of the isoform-specific antibodies. We chose Ramos cells since the cytoplasmic TFII-I isoforms were readily detectable in this cell line. Because the level of TFII-I is more in nuclear extract compared with cytoplasmic extract, the extracts were first normalized for the β-isoform by Western blot (Fig. 1C, lanes 1 and 2). The anti-β antibody was then employed to immunoprecipitate the putative complex from normalized amounts of cytoplasmic and nuclear extracts. The blot was first probed with an anti-α antibody (upper panel), followed by anti-β (middle panel) and anti-TFII-I (lower panel). Although the preimmune control was incapable of bringing down any of the isoforms (lane 3), the anti-β antibody was capable of bringing down not only the β- but also the α- and the Δ-isoforms from both cytoplasmic and nuclear extracts (Fig. 1C, lanes 4 and 5), suggesting that these isoforms exist as a complex. However, although we started out with identical amounts of cytoplasmic and nuclear α and β, the amount of precipitated nuclear α and β were 2-fold higher than their cytoplasmic counterparts. Furthermore, the amount of Δ co-precipitated with α and β was sub-stoichiometric, suggesting that the vast excess of Δ-isoform in this cell line is not in a complex with either α- or β-isoforms. Similar results were
obtained from different human and murine cell lines, suggesting that the complex formation is a cell-type-specific and species-independent phenomenon (data not shown). Taken together these results suggest that either the complex formation leads to preferential nuclear localization, that the complex was more stable in the nucleus, or that the isoforms were more readily available to form a complex in the nucleus compared with the cytoplasm. Furthermore, at present we do not know whether the uncomplexed cytoplasmic isoforms remain as monomers, homo-oligomers, or heteromers with other proteins.

Identification of Nuclear Localization Signal (NLS) in TFII-I

 Isoforms of TFII-I

Fig. 1. Isoforms of TFII-I: in vivo complex formation. A, the schematics of TFII-I isoforms. The closed boxes represent the direct repeats denoted as R1-R6, and the open boxes a or b represent the additional amino acids (aa) encoded by either exon A (GPSETD-VDEKQPLSKPLAQ) or exon B (DISTQVPSSETSDPEVEYTLIE) (the peptides used to raise isoform-specific antibodies are underlined). B, Western blot analyses to show the ectopic expression and specificity of isoform-specific anti-TFII-I antibodies. Equal amounts (5 ng) of ectopically expressed and TALON column-purified fractions of either vector alone (lane 1) or the isoforms of TFII-I (lanes 2–5) were compared by Western blotting with an antibody (anti-TFII-I) that recognizes all the four isoforms (left panel). The blot was stripped and reprobed with a peptide antibody raised against either the TFII-I isoform (anti-TFII-1a, middle panel) or the TFII-Ij isoform (anti-TFII-Ij, right panel). C, immune complex of TFII-I from Ramos cytoplasmic (Cyto) and nuclear (Nuc) extracts. For Western blots (lanes 1 and 2), the amount of cytoplasmic (50 μg) and nuclear (25 μg) extracts were adjusted for the TFII-Ij isoform (lanes 1 and 2). Cytoplasmic (75 μg) or nuclear (37.5 μg) extracts were subjected to immunoprecipitation with a pre-immune serum (lane 3) or with an anti-TFII-I antibody (lanes 4 and 5). The immunocomplexes were probed first with an anti-TFII-I antibody (top panel) and reprobed with an anti-TFII-I antibody (middle panel) followed by anti-TFII-I antibody (bottom panel). The positions of the α, β, (128 kDa), α′(-120 kDa), and the Δ (-120 kDa) isoforms are indicated. The α′ band could arise either due to degradation of α or could be a related isoform.

Isoforms—TFII-I has been shown to be distributed between the nucleus and cytoplasm, although the nuclear fraction predominates in most cells (7). To better understand the subcellular localization of TFII-I and its isoforms, we first determined the consensus localization signals in TFII-I (Δ) isoform by computer analysis using the PSORT program (20). Two potential NLSs were identified; the first one, termed NLS2, was present between amino acids 278 and 284, whereas the second one, termed NLS2, was present between amino acids 629 and 632. To test the role of these sequences, we deleted them one at a time and analyzed the expression and subcellular localization of TFII-I in COS cells after tagging with GFP (21). Although GFP alone when co-expressed with the vector control did not exhibit any specific localization (expressed equally in both cytoplasm and nucleus) (Fig. 2A, extreme left panel), both wild type TFII-IΔ (middle left panel) and the NLS2 mutant, ΔNLS2 (extreme right panel), exhibited predominant nuclear localization with very small amounts in the cytoplasm. In contrast the NLS1 mutant, ΔNLS1 (middle right panel), showed exclusively cytoplasmic fluorescence. Hence we conclude that NLS1 is the predominant and functional NLS under the conditions tested and in this cell type. This is also confirmed by generation of a double mutant of both NLS1 and NLS2 that showed exclusively cytoplasmic localization (data not shown).

Given the fact that the NLS1 is present in all isoforms, as a first approximation it was reasonable to assume that all the isoforms would have similar subcellular localization. However, because the extra exons were present very close to NLS1, it was necessary to determine whether they might affect the subcellular localization of these isoforms. To test this, each of the isoforms were GFP-tagged and expressed in COS cells (Fig. 2B). Moreover, in each case, the cells were also stained with DAPI to visualize the nucleus (purple blue fluorescence, middle panels). Each of the TFII-I isoforms were predominantly found in the nucleus under this condition (GFP, top panels). This conclusion is further strengthened by the superimposition of DAPI and GFP images (bottom panels). However, a small fraction of each of these isoforms was expressed in the cytoplasm as well (not shown). Regardless of the exact amount of cytoplasmic and nuclear TFII-I, which appear to differ depending on the cell type and source of cells (data not shown), similar nucleo-cytoplasmic distribution was observed upon the ectopic expression of each of the isoforms. These results strongly suggest that the exons A and B do not significantly alter the nuclear localization of these proteins.

Next we wanted to determine whether these isoforms interact with themselves (homomerization) and with each other (heteromerization) under these conditions. We rationalized that since the ΔNLS1 TFII-I remains in the cytoplasm, it might be used as a bait to test heteromeric interactions and alterations in subcellular localization. Thus, if the mutant interacts with any of the wild type isoforms and exhibits green fluorescence in the nucleus, then the nuclear translocation is dominant. Alternatively, if the cytoplasmic retention is dominant, then the mutant will retain the wild type isoforms in the cytoplasm. To visualize and/or co-localize the partner protein, we employed the α- and β-isoform-specific antibodies. As expected, ΔNLS1, when co-expressed with the vector control, remained in the cytoplasm (Fig. 3, panels A and M), and the anti-β antibody did not show significant levels of endogenous β isoform (panel E). However, upon co-expression with either α-, β-, or γ-isoform (visualized in red, panels F–H), the mutant migrated to the nucleus in each case, as clearly evidenced by the nuclear green fluorescence (compare panels A with B–D).
demonstrating that nuclear migration of ΔNLS1 is correlated to its interaction with the wild type isoforms. Superimposition of GFP, Alexa 594, and DAPI (compare panels M with N–P) further confirmed that the isoforms, upon co-expression, interact with the Δ-isoform, and such interactions lead to nuclear residency.

Homo- and Heteromerization between Various Isoforms—Although the above experiments showed heteromerization between the Δ-and other isoforms, they did not address whether homomerization is also possible and whether the α-, β-, and γ-isoforms would interact with each other. To address this issue, we co-expressed either GFP-tagged version of α-, β-, γ-, or Δ-isoforms together with either a GST-tagged Δ-isoform or its truncation mutant p70 (1) as baits (Fig. 4). A GST pull-down assay was performed and, after the Western blot was visualized with an anti-GFP antibody, the blot was stripped and reprobed with an anti-GST antibody. As a control, only the GST protein was used as a bait and did not pull down the β-isoform (lane 1). Identical results were also obtained with other isoforms (data not shown). The specificity of the anti-GFP antibody was evident, as it failed to recognize GST-tagged Δ-isoform (lane 2). Interaction of truncated p70 and wild type Δ-isoform (lane 3) indicated that homomerization does happen. In accordance with the in vivo data (Fig. 1C), interaction between Δ- and α- (lane 4) or Δ- and β- (lane 5) or Δ- and γ- isoforms (lane 6) occurred. In addition, interactions between the α- and the β-isoform (lane 7) indicated that heteromerization between isoforms can take place. Densitometric scanning revealed that the amount of the interacting partners (GFP-tagged) is proportional to the amount of the pulled down proteins (GST-tagged). Hence when the amount of the various GST-tagged proteins were equalized, the amount of co-precipitating GFP-tagged interacting partners became nearly identical in all cases. Thus, although both homo- and heteromeric interactions within the isoforms occur, the extent of these interactions is similar.

Differential Regulation of Target Genes by Complex Formation—To unambiguously test the DNA binding activities of TFII-I isoforms, we expressed them individually in bacteria. For this purpose each of the cDNAs was subcloned into a hexahistidine-containing pET11-d vector (12). These proteins were expressed in bacteria and affinity-purified over a TALON column (Fig. 5) by virtue of their hexahistidine tag. The purified proteins were visualized by an anti-TFII-I polyclonal antibody that recognizes all the isoforms (Fig. 5C). The DNA binding ability of these expressed proteins was tested by using...
Isoforms of TFII-I

Interactions between ectopically expressed TFII-I isoforms lead to nuclear translocation. Interactions between ectopically expressed TFII-I isoforms lead to nuclear translocation of the NLS1 mutant. COS7 cells were transfected with 600 ng of plasmids encoding either GFP-tagged ΔNLS1 alone (panels A, E, I, and M) or together with TFII-Iα (panels B, F, J, and N), TFII-Iβ (panels C, G, K, and O), or TFII-Iγ (panels D, H, L, and P), and the green fluorescence was visualized (panels A–D). Ectopically expressed TFII-Iα, TFII-Iβ, and TFII-Iγ isoforms were visualized by indirect immunofluorescence with either protein A-Sepharose-purified anti-TFII-Iα (panel F) or anti-TFII-Iβ (panels E, G, and H) antibody. Nuclei were stained with DAPI (panels I–L). Superimposition (Merge) of GFP, Alexa 594, and DAPI staining are shown in the bottom panels (M–P). Images were obtained using a fluorescent microscope (Nikon, E400) with 100× objective.

Fig. 3. Interactions between ectopically expressed TFII-I isoforms lead to nuclear translocation. Interactions between ectopically expressed TFII-I isoforms lead to nuclear translocation of the NLS1 mutant. COS7 cells were transfected with 600 ng of plasmids encoding either GFP-tagged ΔNLS1 alone (panels A, E, I, and M) or together with TFII-Iα (panels B, F, J, and N), TFII-Iβ (panels C, G, K, and O), or TFII-Iγ (panels D, H, L, and P), and the green fluorescence was visualized (panels A–D). Ectopically expressed TFII-Iα, TFII-Iβ, and TFII-Iγ isoforms were visualized by indirect immunofluorescence with either protein A-Sepharose-purified anti-TFII-Iα (panel F) or anti-TFII-Iβ (panels E, G, and H) antibody. Nuclei were stained with DAPI (panels I–L). Superimposition (Merge) of GFP, Alexa 594, and DAPI staining are shown in the bottom panels (M–P). Images were obtained using a fluorescent microscope (Nikon, E400) with 100× objective.

a radiolabeled probe containing either the Inr sequence derived from the Vβ promoter (Ref. 3 and Fig. 5A) or a TFII-I binding site overlapping the SRE derived from the c-fos promoter (Ref. 3 and Fig. 5B). With each isoform, a roughly similar mobility shifted complex was observed that was ablated by the affinity-purified TFII-I antibody, confirming the authenticity of the protein binding to the probe. When measured by densitometric scanning, the extent of Vβ Inr binding by TFII-Iα, β, and γ isoforms relative to Δ- were as follows: α (3.65), β (0.64), and γ (1.42). Similar results were also obtained with the SRE probe, and the specificity of their binding was also very similar to that of TFII-IΔ (data not shown).

We then tested the transactivation potentials of these isoforms both individually and in combinations on both Vβ and c-fos promoters (Fig. 6). The Vβ promoter was chosen to study the basal transcriptional activity of the TFII-I isoforms and (3), and the c-fos promoter was chosen to study its signal-responsive activation potentials, since TFII-I hyperactivates the c-fos promoter in response to serum and various growth factor stimulation (2, 3). All the individual isoforms showed comparable transcriptional activities (3-3.5-fold) on the Vβ promoter compared with the vector only control (Fig. 6A, compare lanes 1 with 2–5). However, the basal activity was further enhanced by about 2-fold when the isoforms were co-expressed. The α+Δ combination (lane 6) was the most active, but the combinations of Δ+β (lane 7) and α+β (lane 8) was also more active than the individual isoforms. The comparable expression of the isoforms, either singly or in combination, was demonstrated by Western blot analysis, although a slightly less expression of Δ+β combination was seen (Fig. 6A, bottom panel). In contrast to the Vβ promoter, both the individual and the combinations of isoforms showed quite different activities on the c-fos promoter in response to EGF stimulation (Fig. 6B). The Δ-isofor was nearly 2-fold more active compared with the γ isofor, whereas α and β isoforms were 65% as active as the Δ-isofor. Moreover, the combination of isoforms showed 20–40% less activity compared with the individual isoforms. The basal activity (EGF) did not change significantly in any lane. Once again the Western blot analysis showed that the expression levels of these transfected proteins were comparable either in the presence or in the absence of EGF (Fig. 6B, bottom panel).

The functional assays were done such that the ectopic expression of isoforms was maintained at an equivalent level. If the assays were normalized with respect to the relative DNA binding activities of the isoforms, the differences between the promoters might be even greater. Nevertheless, these functional data suggest that homomer versus heteromer formation differentially regulates the transcriptional activity of TFII-I on different promoters.

DISCUSSION

The signals generated outside a cell are transduced to the nucleus through a series of complicated biochemical steps, ultimately resulting in spatial and/or temporal activation of specific sets of genes. Thus, there must exist specific proteins to direct signal transduction pathways to cell type-specific genes and, thereby, provide a molecular link between signal transduction and developmental/differentiation programs in a given cell. Transcription factors play a critical role in development and differentiation in general and often serve as links between signal transduction (origin) and cell type-specific gene activation (end point). Included in this group of transcription factors
Isoforms of TFII-I

are the Rel family of factors (22, 23), signal transducers and activators of transcription (STAT) family of factors (24), and the nuclear factor of activated T cells (NFAT) family of factors (25). Recent data from our laboratory and others suggest that TFII-I is functionally included in this group of factors that link signal transduction events to transcription (2, 3, 5, 7, 13).

Why are there four isoforms of TFII-I apparently with similar DNA binding and transcriptional activities? Do they indeed have redundant functions, or might it be that they have cell type-specific expression and function? Of the four isoforms, the γ-isoform is perhaps expressed predominantly, if not exclusively, in neuronal cells (8). In our analysis of lymphoid and other non-neuronal cells, we have not observed the expression of the γ-isoform clearly by Western blot analysis. However, in our preliminary Western blot analysis of PC12 cells, a protein of approximately 150 kDa (corresponding to apparent molecular mass of the γ-isoform) cross-reacts with the anti-TFII-I antibody (data not shown). It remains to be proven whether this band indeed corresponds to the γ-isoform.

Our preliminary data also indicate the presence of at least two other cell type-specific isoforms.2 We have consistently seen in Western blot analysis of several cell lines and primary cells a doublet corresponding to 120 and 128 kDa (4, 6, 7). In retrospect, given the reactivity of the antibody and the nearly identical molecular weights and the migration patterns of the α- and β-isoforms, it was impossible to determine whether the 128-kDa isoform represented only one of the isoforms or a combination of both. Now with the generation of the isoform-specific antibodies, we are in a position to distinguish between the expression patterns of α- versus the β-isoforms in various cell types.

We show that the different isoforms of TFII-I interact with each other when co-expressed ectopically or when present endogenously. Interestingly, interactions between different isoforms are also seen with Ikaros protein (26). However, in our case, we do not yet know the precise stoichiometry of these interactions, i.e. whether they form dimers or higher oligomers. Thus, we have maintained the term homomer for interactions between identical isoforms and heteromer for interactions between different isoforms. Regardless of the exact stoichiometry, heteromeric complexes are preferentially found inside the nucleus relative to the cytoplasm. This may be due to the fact that either complex formation facilitates nuclear entry or the heteromeric complex is more stable in the nucleus. However, we do not yet know whether both homo- and heteromeric complexes translocate to the nucleus to the same rate and extent. Moreover, although the “uncomplexed” forms appear to be more in the cytoplasm, from our current data we cannot conclude that homomerization is facilitated in this compartment since the uncomplexed form may not be in a homomeric state and may be in a heteromeric complex with another cytoplasmic component (Fig. 7). In addition, the stability and/or the extent of homomerization of different isoforms may also be different.

Apart from nuclear translocation, which both homomeric and heteromeric complexes can potentially undergo, we surmised that perhaps heteromeric complexes can mediate differential

---

2 V. Cheriyath and A. L. Roy, unpublished information.
gene regulation. Consistent with our expectations, we observed that different combinations of isoforms activate different reporter genes to varying extents. Thus, although the $\Delta + \alpha$ heteromeric combination activates the V$\beta$ basal promoter 2-fold better than either of the individual isoforms, the same combination was 20% less effective than the $\Delta$- and marginally more...
effective than the α-isofrom in mediating signal-induced activation of the c-fos promoter. Because TFII-I is downstream of a variety of extracellular signals (7), an obvious possibility raised by our result is that the different complexes or subcomplexes mediate different signals and, consequently, activate different sets of genes (Fig. 6). It is also likely that complex formation may modulate the amplitude or duration of the signals via differential phosphorylation of the isoforms, although we have not yet assessed the phosphorylation status of the isoforms in the complex in response to distinct signals. Such a mechanism to regulate nuclear import and subsequent function has been described for a variety of proteins (reviewed in Ref. 27). Might there be different subcomplexes in different cell types or species? We believe so. The α-isofrom is not predicted to be expressed in mouse cells (28), and by Western blot analysis we do not see the existence of the α-isofrom in mouse cell lines (data not shown). Thus, in addition to differential regulation of genes in a given cell type, complex formation by TFII-I isoforms might also mediate cell type or species-specific gene regulation. Further analysis is under way to resolve these issues.

Acknowledgments—We thank Joe Lomakin for helping with the figures and Peter Brodeur for critical reading of the manuscript and helpful comments. We are also grateful to the rotating students Xi Chen and Pradip Bista for their contributions. We are particularly grateful to the anonymous reviewer whose thoughtful suggestions considerably improved the manuscript.

REFERENCES

1. Cheriyath, V., Novina, C. D., and Roy, A. L. (1998) Mol. Cell. Biol. 18, 4444–4454
2. Grueneberg, D. A., Henry, R. W., Brauer, A., Novina, C. D., Cheriyath, V., Roy, A. L., and Gilman, M. (1997) Genes Dev. 11, 2482–2493
3. Kim, D.-W., Cheriyath, V., Roy, A. L., and Cochran, B. H. (1998) Mol. Cell. Biol. 18, 3310–3320
4. Manzano-Winkler, B., Novina, C. D., and Roy, A. L. (1996) J. Biol. Chem. 271, 12076–12081
5. Novina, C. D., Cheriyath, V., and Roy, A. L. (1998) J. Biol. Chem. 273, 33445–33448
6. Novina, C. D., Cheriyath, V., Denis, M. C., and Roy, A. L. (1997) Methods 12, 254–263
7. Novina, C. D., Kumar, S., Bajpai, U., Cheriyath, V., Zang, K., Pillai, S., Wortis, H. H., and Roy, A. L. (1999) Mol. Cell. Biol. 19, 5014–5024
8. Perez-Juando, L. A., Wang, Y-K., Peoples, R., Coloma, A., Cruces, J., and Francke, U. (1998) Hum. Mol. Genet. 7, 325–334
9. Roy, A. L., Malik, S., Meisterernst, M., and Roeder, R. G. (1993) Nature 365, 355–359
10. Roy, A. L., Meisterernst, M., Pognonec, P., and Roeder, R. G. (1991) Nature 354, 245–248
11. Wu, Y., and Patterson, C. (1999) J. Biol. Chem. 274, 3207–3214
12. Roy, A. L., Du, H., Gregor, P. D., Novina, C. D., Martinez, E., and Roeder, R. G. (1997) EMBO J. 16, 7091–7104
13. Yang, W., and Desiderio, S. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 604–609
14. Rawlings, D. J., Safran, D. C., Tsukada, S., Largaespada, D. A., Grimaldi, J. C., Cohen, L., Mohr, R. N., Bazan, J. F., Howard, M., Copeland, N. G., Jenkins, N. A., and Witte, O. N. (1993) Science 261, 358–361
15. Tsukada, S., Safran, D. C., Rawlings, D. J., Parolini, O., Allen, R. C., Klosak, I., Sparkes, R. S., Kubagawa, H., Mohandas, T., Quan, S., Belmont, J. W., Cooper, M. D., Conley, M. E., and Witte, O. N. (1993) Cell 72, 279–290
16. Vetrie, D., Vorechovsky, I., Sideras, P., Holland, J., Davies, A., Flinter, F., Hammarstrom, L., Kinnon, C., Levinsky, R., Borew, M., Smith, C. I., and Bentley, D. R. (1993) Nature 366, 221–233
17. O'Mahony, J. V., Guven, K. L., Lin, J., Joya, J. E., Robinson, C. S., Wade, R. P., and Hardeman, E. C. (1998) Mol. Cell. Biol. 18, 6641–6652
18. Osborne, L. R., Campbell, T., Daradich, A., Scherer, S. W., and Tsui, L.-C. (1999) Genes Dev. 13, 279–284
19. Tanaka, M., Gupta, R., and Mayer, B. J. (1995) Mol. Cell. Biol. 15, 6829–6837
20. Nakai, K., and Kanehisa, M. (1992) Genomics 14, 897–911
21. Chaffie, M., Tu, Y., Euskirchen, G., Ward, W. W., and Prasher, D. C. (1994) Science 263, 802–805
22. Baldwin, A. S. (1996) Annu. Rev. Immunol. 14, 649–683
23. Ghosh, S. (1999) Immunol. Rev. 163, 183–189
24. Darnell, J. E. (1997) Science 277, 1630–1635
25. Rao, A., Luo, C., and Hogan, B. (1997) Annu. Rev. Immunol. 15, 767–747
26. Sun, L., Liu, A., and Georgopoulos, K. (1996) EMBO J. 15, 5358–5369
27. Vandromme, M., Gauthier-Rouviere, C., Lamb, N., and Fernandez, A. (1996) Annu. Rev. Immunol. 14, 59–94
28. Wang, Y. K., Perez-Juando, L. A., and Francke, U. (1998) Genes Dev. 12, 163–170
29. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) Methods Enzymol. 11, 1475–1489
