The transcription factor TFII-I binds to distinct promoter sequences including an initiator element in several eukaryotic genes. Here we demonstrate that TFII-I is phosphorylated in vivo at serine/threonine and tyrosine residues in the absence of any apparent extracellular signals. This “basal” phosphorylation of TFII-I is not required and does not affect its specific DNA binding, but is critical for its in vitro transcriptional properties via the Vβ promoter. To better assess the functional role of phosphorylation in regulating TFII-I activity, we focused on tyrosine phosphorylation of TFII-I. Ectopically expressed recombinant TFII-I, like its native counterpart, exhibits tyrosine phosphorylation in the absence of distinct extracellular signals. More important, mutation of a potential consensus tyrosine phosphorylation site in TFII-I leads to severe reduction in its basal transcriptional activation of the Vβ promoter in vivo. Taken together, these data suggest that tyrosine phosphorylation of TFII-I is important for its initiator-dependent transcriptional activity.

TFII-I is a multifunctional transcription factor with broad biochemical and biological activities and may be involved in several genetic disorders (1–6). Therefore, it is important to undertake biochemical dissection of TFII-I to gain functional insights into this novel protein. We have previously shown that TFII-I can bind to the Inr element in the natural TATA-less but Inr-containing T-cell receptor Vβ promoter and is required for its Inr-dependent transcription in vitro (4) and in vivo (7). TFII-I can also bind specifically to the Inr element in the TATA- and Inr-containing adenovirus major late promoter and, together with the upstream stimulatory factor, can markedly enhance adenovirus major late promoter transcription via the Inr element in vivo (6). However, the function of TFII-I is not necessarily restricted to the Inr element, as TFII-I can bind to an upstream regulatory element (E-box) both independently and synergistically with the upstream stimulatory factor and stimulate transcription in vivo through E-box elements in the absence of an Inr (6). Thus, TFII-I may serve as a transcriptional cofactor that potentially integrates signals from the regulatory components to the basal machinery. Consistent with its multifunctional potentials, TFII-I promotes formation of serum response factor and homeodomain protein Phox1 complexes on the c-fos promoter at the upstream serum response element (8). TFII-I functions through the upstream c-sis-inducible factor element and serum response elements, and ectopic expression of TFII-I leads to enhanced transcriptional activation of the c-fos promoter to a variety of stimuli (e.g. epidermal growth factor, platelet-derived growth factor, and 12-O-tetradecanoylphorbol-13-acetate) (9). Furthermore, growth factor stimulation leads to increased tyrosine phosphorylation of TFII-I (9). Finally, TFII-I is identical to the recently discovered protein BAP-135, which is associated in vivo with Btk (Bruton’s tyrosine kinase), a mutation that leads to X-linked immune deficiency in humans and mice (10). TFII-I is tyrosine-phosphorylated by Btk in vitro and upon immunoglobulin receptor cross-linking in B-cells (10). These observations suggest that TFII-I is a novel factor that can link signal-responsive activator complexes to the general transcription machinery perhaps in a cell type-dependent fashion. Interestingly, TFII-I has also been identified in the breakpoint regions of the 7q11.23 Williams-Beuren syndrome deletion (11).

To begin to understand the specific role that phosphorylation plays in regulating the functional activity of TFII-I, we have undertaken biochemical analysis of this multifunctional protein. Here we show that TFII-I is phosphorylated basally (i.e. in the absence of apparent extracellular signals) at serine/threonine and tyrosine residues. In vitro dephosphorylation of native TFII-I suggests that phosphorylation is dispensable for its specific DNA binding activity, but is required for its transcriptional activity. Extending these studies in vivo, we show that compared with wild-type TFII-I, a mutant TFII-I (in which the consensus tyrosine phosphorylation site is changed to phenylalanine) failed to significantly activate the Vβ promoter. Thus, tyrosine phosphorylation of TFII-I is critical for its basal transcriptional properties.

EXPERIMENTAL PROCEDURES

In Vivo Labeling—In vivo labeling of cultured cells with inorganic phosphate and subsequent processing were done essentially as described (12). HeLa cells (3 x 10⁶/ml x 66 ml) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% newborn calf serum and harvested. Cell pellets were washed and resuspended in serum-free and phosphate-free Dulbecco’s modified Eagle’s medium. The resuspended cells were supplemented with 1 mCi of [32P]orthophosphate (3000 Ci/mmol). Labeling proceeded for 4 h at 37 °C.

Immunoprecipitation and Phosphoamino Acid Analysis (PAA)—Phosphate-labeled cells were harvested and washed in ice-cold Tris-buffered saline (3 x 10 ml), and nuclear extracts were prepared (13). Anti-TFII-I antiserum (5 μl) was coupled to protein A-agarose (15 μl, 1:1 slurry) in buffer A (20 mM Tris (pH 7.9 at 4 °C), 0.2 mM EDTA, 5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10% glycerol, and 100 mM KCl) containing 0.05% Nonidet P-40, incubated for 40 min at 4 °C, washed in buffer A with 0.05% Nonidet P-40 and 12-O-tetradecanoylphorbol-13-acetate (9). Furthermore, growth factor stimulation leads to increased tyrosine phosphorylation of TFII-I (9).

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The beads were washed in buffer A containing 0.05% Nonidet P-40, resuspended in Laemmlı sample buffer, and subjected to 7.5% SDS-PAGE. The gel was Cooamassie Blue-stained, dried, and briefly subjected to autoradiography. A band corresponding to 120 kDa was excised from the gel, rehydrated, and subjected to protease inhibitors 4-(2-aminoethyl)benzenesulfonyl fluoride, aprotinin, phenylmethylsulfonfyl fluoride, and soy bean trypsin inhibitor. The cells were incubated on ice for 5 min with periodic vortexing and centrifuged for 10 min at 14,000 rpm and 4 °C.

**RESULTS**

**TFII-I Is Phosphorylated in Vivo**—A highly interesting feature revealed by analysis of the primary amino acid sequence of TFII-I is the presence of a number of potential phosphorylation sites, including 35 potential serine/threonine phosphorylation and two tyrosine phosphorylation (EDXYD) sites, suggesting that TFII-I has the potential to be differentially regulated in response to distinct physiological stimuli (6). Thus, we first tested the phosphorylation status of native TFII-I in HeLa cells (Fig. 1) by PAA. TFII-I was immunoprecipitated by a highly specific anti-TFII-I antibody from orthophosphate-labeled HeLa cells and subjected to SDS-PAGE and autoradiography (Fig. 1A). The band corresponding to 120 kDa/TFII-I was excised and subjected to PAA (Fig. 1B). When compared with the ninhydrin standards, PAA revealed that TFII-I was phosphorylated predominantly at serine residues. These assays employed acid hydrolysis of peptide bonds. Because phosphothreonine and phosphotyrosine are acid-labile relative to phosphoserine (14), alternative methods were employed to detect potential phosphorylated threonine and tyrosine residues in TFII-I. The detection of phosphotyrosine residues in HeLa-derived TFII-I was attempted by immunochromatography subsequent to immunoprecipitation with anti-phosphotyrosine antibody. Because TFII-I phosphorylation decreases upon purification, a two-column-purified fraction containing TFII-I derived from HeLa nuclear extracts was used for this analysis (Fig. 1C). TFII-I was detected in immunoprecipitates from this fraction with a monoclonal anti-phosphotyrosine antibody, but not with protein A-Sepharose beads alone. However, the amount of TFII-I in the immunoprecipitate, compared with the supernatant, was low (data not shown), indicating either that TFII-I is weakly phosphorylated at tyrosine residues or that only a fraction of total TFII-I is tyrosine-phosphorylated basally under these conditions.

**Role of Phosphorylation in in Vitro Function of TFII-I**—We tested whether the basal phosphorylation of TFII-I is necessary and sufficient for its DNA binding and transcription functions. To determine the functional role of phosphorylation of TFII-I, we dephosphorylated native TFII-I in vitro and tested its DNA binding (Fig. 2) and transcriptional (Fig. 3) properties. EMSA showed that the dephosphorylated protein (Fig. 2A, lanes 2–4)
was capable of binding to the Vβ Inr probe almost as efficiently as its phosphorylated counterpart (lane 1). At the highest phosphatase concentrations (lane 4), there was a slight reduction in DNA binding. To demonstrate that the recovery of TFII-I protein after phosphatase treatment was nearly identical in all lanes, Western blot analysis with anti-TFII-I antibody was performed (Fig. 2B, left panel). The slight reduction in EMSA (Fig. 2A, lane 4) corresponds to a lower recovery of protein observed in Western blotting with anti-TFII-I antibody (Fig. 2B, left panel, lane 4). To demonstrate that TFII-I was efficiently dephosphorylated under these conditions, the blot was stripped and reprobed with anti-phosphoserine antibody (Fig. 2B, right panel). Untreated native TFII-I (lane 1), but not phosphatase-treated TFII-I (lanes 2–4), was specifically immunoreactive to anti-phosphoserine antibody, suggesting that the dephosphorylation reaction proceeded to completion to the limit of detection by this analysis. Identical results have also been obtained with anti-phosphothreonine and anti-phosphotyrosine antibodies (data not shown). Next we tested whether the DNA binding specificity of TFII-I was altered during dephosphorylation and found that it was indistinguishable from that of the corresponding phosphorylated protein (Fig. 2C). Taken together, these results demonstrate that phosphorylation of TFII-I does not significantly alter its DNA binding properties either qualitatively or quantitatively. Finally, we tested the effect of dephosphorylation of TFII-I on Vβ transcription in Jurkat nuclear extracts immunodepleted of endogenous TFII-I (Fig. 3A, compare lanes 1 and 2). Although native phosphorylated TFII-I was competent to fully restore Vβ transcription (compare lanes 2 and 3), identical amounts of in vitro dephosphorylated TFII-I were incompetent to restore Vβ transcription when added to the TFII-I-depleted extract (compare lanes 3 and 4), indicating that phosphorylation of TFII-I is required for its transcriptional activity. To demonstrate that the lack of transcriptional activity of dephosphorylated TFII-I under our assay conditions is not due to carryover of residual phosphatase leading to nonspecific inhibition of the transcription reaction, control experiments were performed (Fig. 3B). Vβ transcription (lane 1) was not inhibited by addition of either bovine serum albumin alone (lane 2) or alkaline phosphatase-treated bovine serum albumin (lane 3).

Tyrosine Phosphorylation of Ectopic TFII-I in Vivo—TFII-I has been recently cloned by us and by others as a multifunctional transcription factor (6, 8) as well as a protein (BAP-135) that associates with Bruton’s tyrosine kinase, mutations that are associated with X-linked agammaglobulinemia in humans and X-linked immunodeficiency in mice (10). We employed a recombinant form of TFII-I (GST-TFII-I/p146) (7–9) to study its

**Fig. 1.** TFII-I is serine- and tyrosine-phosphorylated in vitro. A, immunoprecipitates of TFII-I, from orthophosphate-labeled HeLa cells with anti-TFII-I antibody, when subjected to SDS-PAGE and autoradiography, demonstrated a heavily labeled protein that migrated with a relative molecular mass of 120 kDa, identical to native TFII-I. B, PAA of the excised band in A demonstrated that the 120-kDa band TFII-I is phosphorylated at serine residues in vitro. Ninhydrin-stained spots are marked as follows: Y, phosphotyrosine; T, phosphothreonine; S, phosphoserine; Ori, origin; P, free phosphate. The directions for electrophoresis and chromatography are marked. C, Western blotting with anti-TFII-I antibody (α-TFII-I) indicated the presence of TFII-I in an anti-phosphotyrosine precipitate (α-P-Tyr ppt.), but not in a control precipitate (Control ppt.), of partially purified TFII-I fractions derived from HeLa nuclear extracts.

**Fig. 2.** Phosphorylation of TFII-I is dispensable for DNA binding. Highly purified TFII-I was dephosphorylated in vitro with increasing amounts of alkaline phosphatase-coupled agarose beads (Ptpase). One-fourth of each reaction was loaded in EMSA (A), and three-fourths of the reaction was loaded in SDS-PAGE (B). A, phosphorylated TFII-I (lane 1) bound the Vβ Inr-containing probe to the same extent as phosphatase-treated TFII-I (lanes 2–4). B, phosphorylated (lane 2) and phosphatase-treated (lanes 2–4) TFII-I subjected to SDS-PAGE was blotted and probed first with anti-TFII-I antibody (α-TFII-I). This blot was stripped and reprobed with anti-phosphoserine antibody (α-P-Ser). C, dephosphorylated TFII-I bound with the same specificity as the phosphorylated protein. Binding of the dephosphorylated protein to the Vβ Inr-containing probe was competed with wild-type Inr-containing (W), but not with mutant Inr-containing (M) or E-box-containing (U), oligonucleotide competitor (Comp).
function and phosphorylation status in mammalian cells (COS-7) since these cells express low amounts of endogenous TFII-I (7). To test whether recombinant TFII-I, like native TFII-I, exhibits tyrosine phosphorylation, COS-7 cells ectopically expressing p146 were treated with pervanadate (a potent tyrosine phosphatase inhibitor) (18), and the derived lysates were analyzed by Western blot analysis using a monoclonal anti-Tyr(P) antibody (Fig. 4A, left panel). Compared with both the untransfected and transfected control lysates in the absence of pervanadate, the pervanadate-treated transfected lysate demonstrated several proteins with markedly increased phosphotyrosine content. This blot was stripped and reprobed with anti-TFII-I antibody (Fig. 4A, right panel) to demonstrate that comparable amounts of p146 are expressed in both pervanadate-treated and untreated lysates. To ensure that p146 is among the proteins that exhibit increased tyrosine phosphorylation, it was pulled down with GST-agarose and probed with anti-Tyr(P) antibody (Fig. 4B, left panel). Whereas beads from neither the untransfected control lysate nor the transfected but untreated lysate showed any significant anti-Tyr(P) antibody-reactive bands, beads from the pervanadate-treated lysate showed a 146-kDa anti-Tyr(P) antibody-reactive band. When stripped and reprobed with anti-TFII-I antibody (Fig. 4B, right panel), the 146-kDa tyrosine-phosphorylated protein comigrated with GST-TFII-I/p146. Moreover, these data show that almost equal amounts of GST-TFII-I/p146 are pulled down in the untreated lysate compared with the pervanadate-treated lysate. In contrast, and as expected, pervanadate treatment did not cause any significant increase in phosphoserine on ectopically expressed GST-TFII-I since TFII-I from both the pervana- date-treated and untreated lysates showed equal anti-phosphoserine reactivity (Fig. 4C, left panel) and equal recovery of p146 under both conditions (right panel). We conclude that ectopically expressed TFII-I exhibits tyrosine phosphorylation in the absence of any apparent extracellular signals. The pervana- date-treated and untreated lysates were also compared in EMSA, and no significant differences were observed (data not shown).

Mutation of a Consensus Tyrosine Phosphorylation Motif in TFII-I Affects Its Transcriptional Activity via the Vβ Promoter—There are two consensus tyrosine phosphorylation motifs (EDXDY) in TFII-I: at positions 244–248 (site I) and 273–277 (site II) (6). Site I additionally has a tyrosine residue at position 249. However, of the two sites, data base (PROSITE) search only recognized site I as a consensus tyrosine phosphorylation site. Hence, we first decided to mutate this site. PCR-directed mutagenesis was employed to generate a mutant in which both tyrosine residues at positions 248 and 249 in site I were altered to phenylalanine. This mutant henceforth is referred to as the Y-F mutant. To compare the transcriptional phenotype of this

**Fig. 3.** Phosphorylation of TFII-I is required for transcription in vitro. A, mock depletion (Mock Depl.) of Jurkat nuclear extract with preimmune antibody (lane 1) did not affect in vitro transcription of the Vβ template. However, transcription was abrogated by depletion of TFII-I (TFII-I Depl.) with anti-TFII-I antibody (lane 2). Vβ transcription was reconstituted by native purified TFII-I (lane 3), but not by dephosphorylated TFII-I (Dephos. TFII-I; lane 4). B, control reactions under identical conditions as in A demonstrated that Vβ transcription (lane 1) is not affected by either bovine serum albumin alone (BSA; lane 2) or alkaline phosphatase-treated bovine serum albumin (Dephos. BSA; lane 3).

**Fig. 4.** Tyrosine phosphorylation of overexpressed TFII-I. Whole cell lysates were prepared from untransfected COS-7 cells or COS-7 cells ectopically expressing GST-TFII-I (146 kDa) with or without 10 min of pervanadate treatment. A, lysates (50 μg) compared by Western blotting with anti-phosphotyrosine antibody (α-P-Tyr; left panel) demonstrated little tyrosine phosphorylation in the untransfected lysate (UTL) and in the p146-transfected lysate in the absence of pervanadate (GST-TFII-I + Perc.) compared with the p146-transfected lysate prepared after pervanadate treatment (GST-TFII-I + Perv.). This blot, when stripped and reprobed with anti-TFII-I antibody (α-TFII-I; right panel), demonstrated no GST-TFII-I in the untransfected lysate, but approximately equivalent amounts of GST-TFII-I in the p146-transfected lysates prepared with and without pervanadate treatment. B, GST pull down was subjected to Western blot analysis with anti-phosphotyrosine antibody (left panel). Analysis of this blot after stripping and reprobing with anti-TFII-I antibody (right panel) confirmed the identity of recombinant TFII-I in the precipitated material. C, a control blot prepared similarly as described for B, when probed with anti-phosphoserine antibody (α-P-Ser; left panel), showed no significant differences in serine phosphorylation between pull down assays of p146-transfected lysates prepared with and without pervanadate treatment.
mutant with that of the wild-type protein, transient transfection assays were performed using a Vβ reporter construct as described (7). Whereas wild-type TFII-I exhibited nearly a 4-fold induction of Vβ promoter activity, the mutant failed to activate the promoter significantly over and above the control levels (Fig. 5, upper panel). The residual activity seen with the mutant could be due to tyrosine phosphorylation of site II, which has not yet been mutated. More important, the difference in transcriptional activity between wild-type and mutant TFII-I is not due to a difference in their protein expression since Western blot analysis showed that the expression levels were very similar, and thus, the Y-F mutation does not cause any apparent protein instability (Fig. 5, lower panel). That the Y-F mutation leads to reduced basal tyrosine phosphorylation of TFII-I was demonstrated by GST pull-down assays (Fig. 6). Lysates were prepared from COS-7 cells transiently transfected with either wild-type or Y-F mutant TFII-I and incubated with GST-agarose. The GST beads were boiled and subjected to SDS-PAGE and Western blot analysis with anti-phosphotyrosine antibody (left panel). The difference in tyrosine phosphorylation signals was quantitated by densitometry and found to be 2.32-fold higher in the wild-type than in the mutant protein. To show that equivalent quantities of wild-type and mutant proteins were loaded, the blot was stripped and reprobed with anti-TFII-I antibody. The level of mutant TFII-I was 1.45-fold higher than wild-type TFII-I, and thus, the difference in tyrosine signals between the wild-type and mutant proteins is nearly 4-fold. However, although the mutant shows reduced tyrosine phosphorylation, it still shows some residual tyrosine phosphorylation, which could be due to site II.

**FIG. 5. Tyrosine phosphorylation of TFII-I is critical for its transcriptional activity in vivo.** Upper panel, COS-7 cells were transiently cotransfected with 350 ng of wild-type Vβ reporter construct (7) and with 200 ng of plasmid expressing wild-type TFII-I (WT), Y-F mutant TFII-I, or vector alone. The transfection efficiencies were normalized by including 35 ng of renilla luciferase expression plasmid in each transfectant (pRL-TK, Promega). The -fold activation was calculated by normalizing the wild-type Vβ promoter activity to 1 (7). The results represent an average of three independent experiments. Lower panel, lysates from transfection assays were subjected to Western blot analysis to show the comparable expression of wild-type and Y-F mutant TFII-I. Equal amounts of cell lysates (6 μg of protein) from each transfection were subjected to Western blot analysis with anti-TFII-I antibody.

**Fig. 6. Y-F mutation leads to reduced tyrosine phosphorylation of TFII-I in vivo.** COS-7 cells were transfected with 10 μg of wild-type (WT) or Y-F mutant TFII-I expression plasmids. Cells were lysed 60 h post-transfection and subjected to GST pull down as described under "Experimental Procedures." GST-Sepharose beads bound to proteins were resolved by 10% SDS-PAGE. The tyrosine phosphorylation levels were detected by Western blot analysis with anti-Tyr(P) antibody 4G10 (α-P-Tyr; left panel). The blot was stripped and reprobed with anti-TFII-I antibody (α-TFII-I; right panel).

**DISCUSSION**

Regulation of gene-specific transcription factor activity by phosphorylation is not unique (19–21). However, direct regulation of core promoter activity via phosphorylation of components of the basal transcription machinery has been only recently demonstrated (22–24). In line with these latter observations, we show here that basal transcription of the TATA-Inv β promoter is regulated via phosphorylation of TFII-I. For these studies, we attempted to determine the role of tyrosine phosphorylation in TFII-I. We demonstrate that tyrosine phosphorylation of TFII-I is required for its Inr-dependent transcriptional activity. To our knowledge, this is the first report that demonstrates basal transcriptional regulation via tyrosine phosphorylation. Although tyrosine phosphorylation of TFII-I is required for its basal transcription function, we do not yet know the precise mechanism. Since the DNA binding seems to be unaffected by phosphorylation, it is likely that the protein-protein interactions of TFII-I with the basal machinery may be dependent upon its phosphorylation status. It is also possible that tyrosine phosphorylation of TFII-I is required for its nuclear translocation. However, the latter does not appear to be the case as the Y-F mutant readily translocates to the nucleus upon ectopic expression (data not shown).

The role of serine phosphorylation in TFII-I is less clear at present. Because there are numerous potential Ser/Thr phosphorylation sites, it is difficult to assess which one of these is utilized inside the cell. It is possible that serine phosphorylation of TFII-I is required for protein stability. Thus, addition of completely dephosphorylated TFII-I to nuclear extract could lead to degradation/proteolysis of the protein. It is also possible that serine phosphorylation may be an obligatory step for tyrosine phosphorylation. Hence, serine phosphorylation may be necessary but not sufficient for transcriptional activity. Since the Y-F mutant retains its serine phosphorylation (data not shown), we cannot rule out this latter possibility. Finally, it remains possible that serine phosphorylation affects its upstream activator functions as opposed to its core promoter functions.

Here we have attempted to analyze the basal phosphoryla-
tion of TFII-I and its potential effects on DNA binding and transcription. Although TFII-I appears to have both core promoter and upstream activator element-dependent transcription functions, use of the basal Vβ promoter does not allow us to assess the importance of the TFII-I phosphorylation for its upstream element-dependent function. Whereas phosphorylation appears to have no significant role in modulating its DNA binding activity, basal phosphorylation seems to be required for its Inr-dependent transcriptional activity. We would like to emphasize that what we termed as basal phosphorylation may not be so since we cannot rule out cell cycle effects and/or effects of growth factors present in serum that are required to grow the cells. However, since TFII-I is tyrosine-phosphorylated even under very low (0.5%) serum conditions (9), it is likely that additional parameters may contribute to phosphorylation of TFII-I. Although we have not yet addressed whether induced tyrosine phosphorylation of TFII-I (e.g. by growth factors) affects its basal transcription functions, induced tyrosine phosphorylation of TFII-I in response to epidermal growth factor stimulation appears to correlate with its upstream element-dependent transcription functions at the c-fos promoter (9). Thus, we postulate that although induced tyrosine phosphorylation of TFII-I may not have any significant role in affecting its DNA binding capabilities, induced tyrosine phosphorylation could have a significant role in modulating its transcription functions, especially upstream activating functions. Because the TFII-I structure reveals several presumptive serine/threonine and tyrosine phosphorylation sites (6), it is likely that TFII-I phosphorylation is induced in response to a wide variety of signaling pathways and consequently affects a multitude of TFII-I-responsive genes. Consistent with this notion, it appears that mitogen-activated protein kinase may be capable of phosphorylating TFII-I both in vitro and in vivo.4 Taken together, these observations suggest that the various functions of TFII-I might be regulated by its differential phosphorylation. Structure-function analysis of TFII-I should provide important clues regarding its presumptive role as a mediator between signal transduction and transcriptional cascades.

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