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Interferon Regulatory Factors and TFIIB Cooperatively Regulate Interferon-Responsive Promoter Activity In Vivo and In Vitro

I-MING WANG,[1] JORGE C. G. BLANCO,[1] SOPHIA Y. TSAI,[2] MING-JER TSAI,[2] AND KEIKO OZATO[1]*

Laboratory of Molecular Growth Regulation, National Institute of Child Health and Human Development, Bethesda, Maryland 20892,[1] and Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030[2]

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Interferon regulatory factors (IRFs) bind to the interferon-stimulated response element (ISRE) and regulate interferon- and virus-mediated gene expression. IRF-1 acts as a transcriptional activator, while IRF-2 acts as a repressor. Here we show that IRF-1 and IRF-2 bind to both cellular TFIIB, a component of the basal transcription machinery, and recombinant TFIIB (rTFIIB) and that this protein-protein interaction facilitates binding of IRFs to the ISRE. A functional interaction between TFIIB and IRF was assessed by a newly established in vitro transcription assay in which recombinant IRF-1 (rIRF-1) stimulated transcription specifically from an ISRE-containing template. With this assay we show that rIRF-1 and rTFIIB cooperatively enhance the ISRE promoter in vitro. We found that the activity of an ISRE-containing promoter was cooperatively enhanced upon cotransfection of TFIIB and IRF-1 cDNAs into P19 embryonal carcinoma cells, further demonstrating functional interactions in vivo. The cooperative enhancement by TFIIB and IRF-1 was independent of the TATA sequence in the ISRE promoter but dependent on the initiator sequence (Inr) and was abolished when P19 cells were induced to differentiate by retinoic acid treatment. In contrast, cotransfection of TFIIB and IRF-1 into NIH 3T3 cells resulted in a dose-dependent repression of promoter activation which occurred in a TATA-dependent manner. Our results indicate the presence of a cell type-specific factor that mediates the functional interaction between IRFs and TFIIB and that acts in conjunction with the requirement of TATA and Inr for promoter activation.

The interferon regulatory factor (IRF) family plays a vital role in gene regulation mediated by interferons (IFNs) and by viral infections (for reviews, see references 10, 42, and 64). The IRF family includes IRF-1, IRF-2, ICSBP, ISGF3γ, and the recently described Pip and IRF-3 (1, 15, 17, 25, 36, 45, 63), all of which share a homologous DNA-binding domain responsible for binding to the interferon-stimulated responsive element (ISRE). IFNs exert pleiotropic activities, including antiviral, immune system-enhancing, and growth-regulatory activities, in a wide variety of cells (for a review, see reference 64). Proteins of the IRF family have been shown to take part in eliciting these activities (43, 46). IRF-1, IRF-2, and ICSBP are constitutively expressed in the nucleus, while ISGF3γ occurs as a latent cytoplasmic protein that is translocated into the nucleus as part of a multisubunit complex formed upon IFN treatment (10, 35). ISGF3 has been shown to be required for early transcriptional responses to IFNs (46). IRF-1 is induced upon IFN treatment and is capable of acting as a transcriptional activator (25, 26, 52). Thus, ectopic IRF-1 expression leads to activation of ISRE promoters in the absence of IFNs. On the other hand, IRF-2 and ICSBP both act as repressors and inhibit IFN-activated transcription (25, 49). While DNA-binding activity has been mapped to the N-terminal domain, the less-conserved C-terminal region has been shown to act as a regulatory domain (25, 68). In addition, proteins of the IRF family interact with each other and with other families of transcription factors (5, 13, 48). These interactions modify ISRE-binding activities of IRF proteins. However, despite recent progress in understanding the diverse activities of the IRF family, the molecular mechanism by which proteins of the IRF family interact with the basal transcription machinery and achieve IFN-mediated gene regulation has not been fully elucidated.

Assembly of the preinitiation complex (PIC), a critical early event in transcription, is a multistep process that requires participation of general transcription factors (GTFs) designated TFIIA through TFIIJ as well as RNA polymerase II (for a review, see reference 70). Sequence-specific activators are thought to stimulate transcription by facilitating the assembly of the PIC (for a review, see reference 59). In accordance, a number of activators have been shown to interact with GTFs, including TATA-box-binding protein (TBP), TBP-associated factors (TAFs), and TFIIB (for a review, see reference 21). TFIIB is a GTF required for transcription from various basal promoters (65). TFIIB is also reported to be involved in RNA polymerase II-dependent transcription that does not require TFIID (62). Earlier, TFIIB was shown to bind to COUP, a member of the nuclear hormone receptor superfamily (61), and an acidic activator, Gal4-AH (38). Since then, a plethora of sequence-specific transcription factors have been reported to interact with TFIIB, notably, viral acidic activators VP16 and EBNA-2 (39, 60); *Drosophila* transcription factors *fushi tarazu* and *krüppel* (9, 54); proteins of the nuclear hormone receptor superfamily, such as thyroid hormone receptor and vitamin D receptor (2, 4, 27, 40); proteins of the Rel/NF-κB family (31, 55, 67); the POU domain-containing protein Oct-1 (47); the CREB coactivator CBP (32); a herpes simplex virus transactivator, ICP4 (22); and a coactivator for human immunodeficiency virus Tat, TAP (69). These reports suggest that TFIIB serves as a bridge between the basal machinery and specific activators. However, with the exception of certain factors (4, 7, 9, 54), the functional significance of these interactions has not been verified. Even with those factors for which evidence of functional interaction has been reported, the nature of the interaction has not been fully understood.

Below we describe the use of several independent protein-

* Corresponding author. Mailing address: Building 6, Room 2A01, Laboratory of Molecular Growth Regulation, National Institute of Child Health and Human Development, Bethesda, MD 20892.

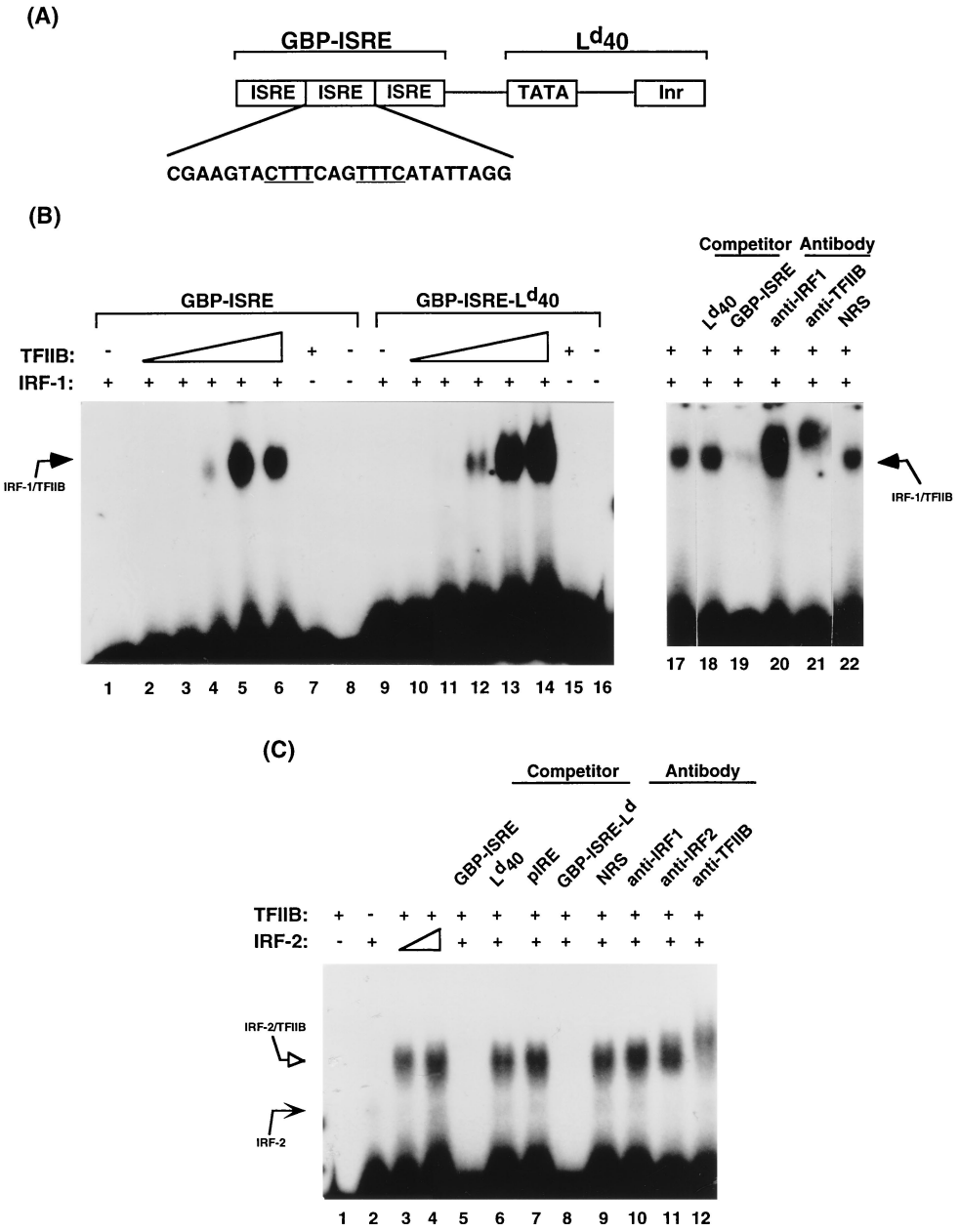

FIG. 1. TFIIB facilitates binding of IRF-1 and IRF-2 to the ISRE. (A) Probes used in EMSA. The GBP-ISRE probe contained three copies of the ISRE. GBP-ISRE-L^{d}40 had a 40-bp basal promoter containing a TATA box and Inr (4, 34). Underlined nucleotides indicate the ISRE consensus core. (B) Left panel: purified rIRF-1 (10 ng), mixed with increasing amounts of rTFIIB (150 ng to 2.4 μg at twofold increments), was probed with ^{32}P-labeled GBP-ISRE (lanes 1 to 8) or with ^{32}P-labeled GBP-ISRE-L^{d}40 (lanes 9 to 16). Right panel: oligonucleotide competitors for GBP-ISRE or basal promoter L^{d}40 were added at a 50-fold molar excess prior to addition of the labeled probe (lanes 18 and 19). Rabbit antibodies against IRF-1 or TFIIB or control normal rabbit sera (NRS) were added at appropriate dilutions prior to addition of the probe (lanes 20 to 22). The IRF-1-TFIIB complex is indicated by arrows. (C) In vitro-translated IRF-2 (0.5 or 1 μl) was added alone or mixed with rTFIIB (2.4 μg) and probed with the GBP-ISRE-L^{d}40 probe (lanes 2 to 4). Oligonucleotide competitors for ISRE or L^{d}40 (described above) or another control, pIRE (a gamma IFN-responsive element unrelated to the ISRE), was added at a 50-fold molar excess (lanes 5 to 8). Rabbit antibodies were added at appropriate dilutions (lanes 9 to 12) as described above. The open-headed arrow indicates the IRF-2–TFIIB complex, whereas the black arrow indicates binding of IRF-2.

protein binding assays to show that physical interactions between IRF-1 (or IRF-2) and TFIIB occur that result in increased ISRE binding. The results of in vitro transcription assays developed in this work support a functional interaction between IRF-1 and TFIIB. A functional analysis in vivo shows that TFIIB exerts a positive or negative effect on ISRE promoter activity that is dependent on cell type. This dual TFIIB activity correlates with the developmental status of cells and with the requirement of TATA and the initiator sequence (Inr) for promoter activity. These results point to the presence of a cell type-specific factor that modulates the interaction between IRFs and TFIIB.

MATERIALS AND METHODS

Plasmids and constructions. Plasmids expressing fusion proteins consisting of glutathione *S*-transferase (GST) fusion proteins and human TFIIB or its deletion mutants, as well as a mammalian expression plasmid, pRS-hTFIIB, were described previously (2, 4). IRF-1 and IRF-2 expression vectors containing the

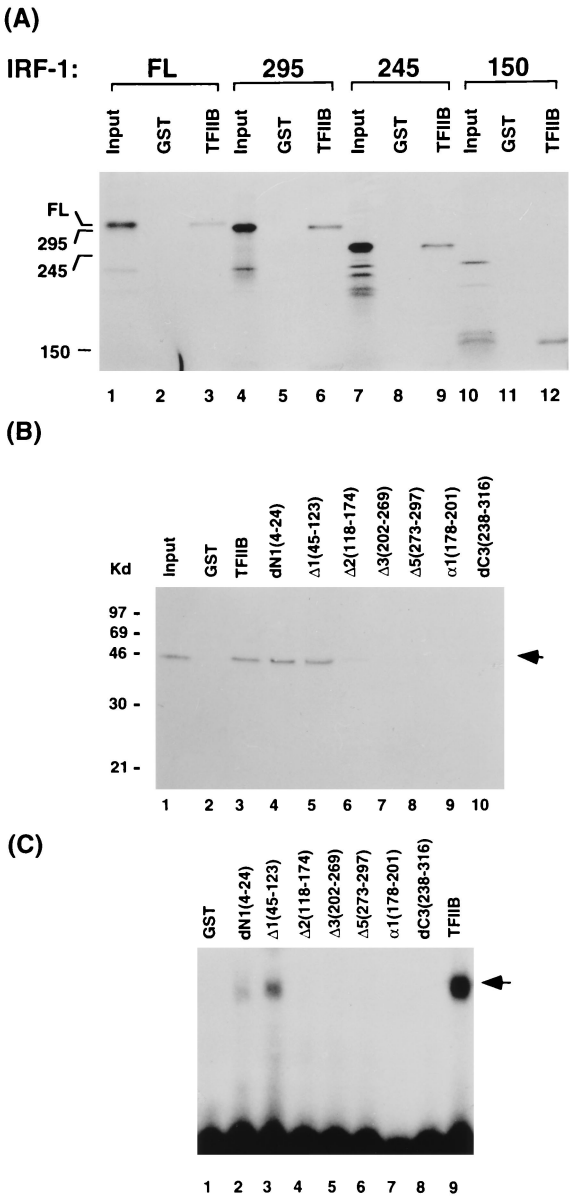

FIG. 2. Binding of IRF-1 to GST-TFIIB: domain analysis. (A) ^{35}S-labeled full-length (FL) and C-terminally truncated IRF-1 (295-, 245-, and 150-amino-acid peptides) were incubated with immobilized GST or GST-TFIIB, and bound materials were analyzed by SDS–10% PAGE (4). The positions of the truncated IRF-1s are marked on the left. (B) ^{35}S-labeled full-length IRF-1 was added to immobilized TFIIB mutants (4, 24) and analyzed as described for panel A. The molecular mass standard positions are shown on the left. The arrow indicates the position of IRF-1. (C) Agarose EMSA was performed for various rTFIIB mutants (2.4 μg) with rIRF-1 (10 ng) as described in the legend to Fig. 1, with GBP-ISRE-L^{d}40 as the probe. The arrow indicates complex formed by IRF-1 and rTFIIB (or rTFIIB mutants).

β-actin promoter (pAct-1 and pAct-2) were gifts from T. Taniguchi (26). The guanylate-binding protein (GBP)-ISRE-L^{d}40 guanine (G)-free template was constructed by cloning three copies of the ISRE from the GBP promoter (GBP-ISRE) as described above into the L^{d}40 G-free cassette containing 377 bp of G-free sequence as described previously (34). Control luciferase reporter pL^{d}40-Luc was constructed by cloning the 40-bp H-2L^d promoter into the pGL basic luciferase plasmid (Promega) (4). The GBP-ISRE-L^{d}40 luciferase reporter was constructed by cloning oligonucleotides corresponding to three copies of the ISRE from the GBP gene (11) into pL^{d}40-Luc as described above. TATA and Inr mutant reporters were constructed by cloning the corresponding mutant oligomers with staggered *Bgl*II (5′)-*Hin*dIII (3′) ends into GBP-ISRE–L^{d}40-Luc from which the basal promoter was removed.

Agarose EMSA and GST-binding assays. Agarose electrophoretic mobility shift assays (EMSAs) were performed according to the method of Lieberman and Berk (37). GBP-ISRE and GBP-ISRE-L^{d}40 probes were generated by digesting the GBP-ISRE–L^{d}40-Luc plasmid with *Xho*I-*Bgl*II and *Xho*I-*Hin*dIII, respectively, and filling in with the Klenow fragment (Bethesda Research Laboratories) in the presence of [α-^{32}P]dCTP (Amersham). IRF-1 cDNA was cloned in a baculovirus vector, pAcSGHisNT (PharMingen), and recombinant IRF-1 (rIRF-1) was prepared from extracts of sf9 cells infected with the recombinant virus by affinity chromatography with Ni-nitriloacetic acid resin (Qiagen). In vitro-translated IRF-1 and IRF-2 were produced from pBS-based plasmids (5) by using a commercially available kit (TNT; Promega). Relative amounts of in vitro-translated IRF-1 and IRF-2 or rIRF-1 and rIRF-2 were estimated from the levels of ^{35}S and/or the intensity of silver staining. rIRF-1 (20 ng) or rIRF-2 (0.5 to 1 μl) was mixed with 0.5 ng of either probe in a buffer containing 12.5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.4), 12.5% glycerol, 5 mM $MgCl_2$, 70 mM KCl, 0.2 mM EDTA, 10 mM 2-mercaptoethanol, 0.5 mg of bovine serum albumin, and 0.6 μg of poly(dC-dG) (Pharmacia) for 45 min in the presence or absence of rTFIIB purified on a glutathione-Sepharose affinity column. Reactions were run in 1.4% agarose gel in G buffer (45 mM Tris-HCl [pH 8.3], 45 mM boric acid, 0.5 mM EDTA, 5 mM magnesium acetate). Rabbit antipeptide antibodies specific for IRF-1 and IRF-2 (5) were added at a 1:100 dilution 15 min prior to addition of the labeled probe. Rabbit antipeptide antibody specific for TFIIB (Santa Cruz Biotechnology) was added at a 1:2 dilution. The GST-binding assay was carried out as described previously (2, 4). Briefly, 10 μg of GST-TFIIB was coupled with 20 μl of glutathione beads and then incubated with in vitro-translated, ^{35}S-labeled IRF-1 at 4°C for 1 h, and bound materials were resolved by sodium dodecyl sulfate (SDS)–10% polyacrylamide gel electrophoresis (PAGE).

Protein-protein binding assay on immobilized DNA beads. The DNA fragment containing three copies of GBP-ISRE was biotinylated at the immediate 5′ sequence of the upper strand (5′-CCAGCGTCCGCAGATCTCCTAAT-3′) by PCR. Twenty picomoles of the biotinylated DNA fragment was immobilized on 1 mg of Dynabeads coupled to streptavidin (M-280 streptavidin; Dynal, Lake Success, N.Y.) in 200 μl of TEN buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8.0) for 1 h at room temperature, with three subsequent washes with TEN buffer (19). The efficiency of conjugation was generally >90%. Conjugated beads were washed in 0.5 ml of TGEDN buffer (20 mM Tris-HCl [pH 8.0], 10% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 100 mM NaCl, and 0.1% Triton X-100) and incubated with purified rIRF-1 (100 ng) and rTFIIB (125 to 500 ng) in a 350-μl reaction mixture containing TGEDN buffer for 1 h at room temperature. Binding assays were performed with nuclear extracts from Namalwa B cells fractionated on a P11 column followed by elution with 0.5 M KCl (51). These materials contained <10 ng of TFIIB as judged by immunoblot analysis. After two washes in TGEDN buffer followed by two additional washes in the same buffer containing 10 μg of poly(dC-dG), bound materials were separated by the magnetic separation procedure according to the instructions of the manufacturer (Dynal). The beads were then heated at 95°C in the solubilizing buffer, and the eluted materials were run on an SDS–10% polyacrylamide gel. Bound IRF-1 and TFIIB were detected by ECL-based immunoblot assays (Amersham).

In vitro transcription reaction. Nuclear extracts were prepared from Namalwa B cells as described by Driggers et al. (14). Nuclear extracts from P19 embryonal carcinoma (EC) cells and NIH 3T3 cells were prepared as follows. Cells were lysed with a buffer containing 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, and 0.5 mM dithiothreitol. Nuclear pellets were incubated in 0.42 M NaCl–20 mM HEPES (pH 7.4)–25% glycerol–1.5 mM $MgCl_2$–0.2 mM EDTA–0.5 mM dithiothreitol for 30 min and then dialyzed. In vitro transcription was carried out as described by Lee et al. (34). In a typical reaction, 200 ng of supercoiled GBP-ISRE-L^{d}40 template and 200 ng of the control L^{d}40 template were mixed with 40 to 160 ng of purified rIRF-1 (or rRXRβ, used as a control) and 25 to 100 ng of rTFIIB and incubated at room temperature for 10 min, followed by addition of 6 to 12 μl of nuclear extract (60 μg of protein) and 1 μg of pBS(+) as a carrier. Reactions were carried out in the presence of [^{32}P]UTP, 0.25 mM 3′-*O*-methyl-GTP, 40 U of RNasin (Promega), and 50 U of RNase T1 (Bethesda Research Laboratories) (14) in a total volume of 25 μl at 30°C for 45 min. The resulting transcripts were analyzed in a 6% acrylamide–urea gel.

Transient transfection. A total of 6×10^4 untreated P19 EC cells or P19 cells treated with all-*trans* retinoic acid (RA) (10^{-6} M; Sigma) (12) for 2 weeks were transfected with 400 ng of the GBP-ISRE-L^{d}40 luciferase reporter and various amounts of either pAct-1 (for IRF-1) or pAct-2 (for IRF-2) and pRSV-hTFIIB by the calcium phosphate precipitation method as described previously (4). Total amounts of transfected DNA were kept constant by including the respective control plasmids without insertions (pRSV-0 or pRSVneo for pRSV-hTFIIB and Lk440 for pAct-1 or pAct-2). NIH 3T3 cells (2×10^5) were transfected with the same reporter and expression vectors by liposome-based transfection using Lipofectamine (Gibco BRL) according to the protocol suggested by the manufacturer. Cells were harvested 24 h after transfection, and luciferase activity was normalized according to the protein concentration. Immunoblot detection of TFIIB and IRF-1 was performed with appropriate antibodies using 10 μg of nuclear extracts prepared from transfected cells as described in reference 4.

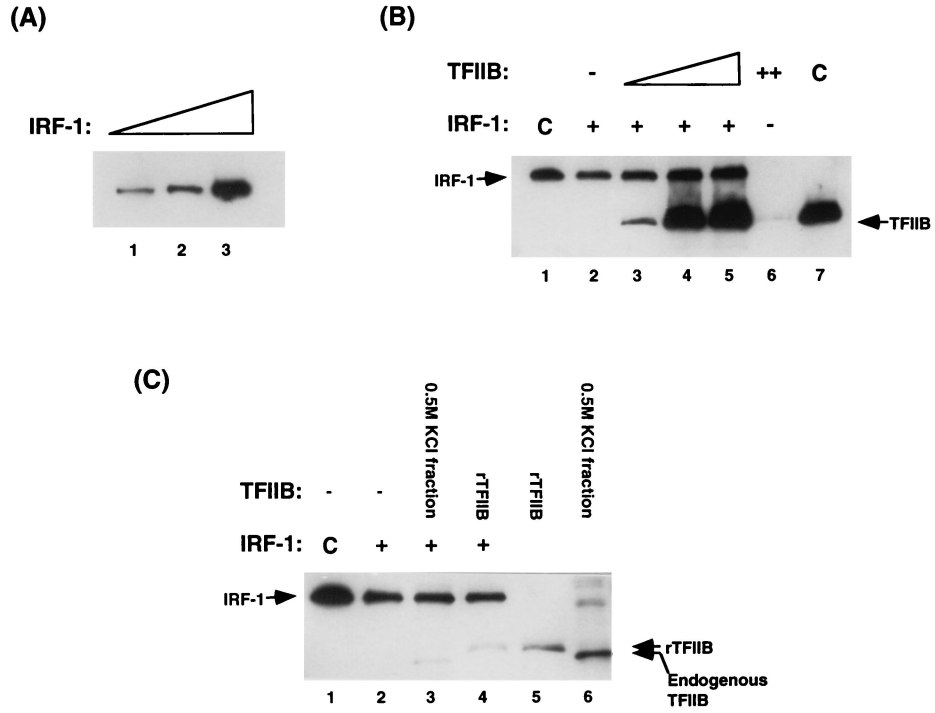

FIG. 3. Binding of TFIIB to rIRF-1 on immobilized ISRE beads. (A) Magnetic beads with immobilized ISRE oligomers were incubated with 10 (lane 1), 20 (lane 2), or 40 ng (lane 3) of rIRF-1. Bound rIRF-1 was estimated by immunoblot analysis. (B) rIRF-1 (100 ng) was incubated with ISRE-immobilized magnetic beads, alone (lane 2) or with increasing amounts of rTFIIB (lanes 3 to 5, with 125, 250, and 500 ng, respectively). Lane 6 contained 500 ng of TFIIB without IRF-1. Lanes 1 and 7 contained 10 ng of rIRF-1 and TFIIB, respectively. Eluted materials were analyzed by immunoblot assays with rabbit antibodies for IRF-1 and TFIIB. (C) Namalwa cell nuclear extracts were fractionated on a P11 column and eluted with 0.5 M KCl. A 100-μl aliquot of each eluate was incubated with ISRE-coated magnetic beads in the presence of 100 ng of rIRF-1; the reaction mixture in lane 4 contained 60 ng of rTFIIB and 100 ng of rIRF-1.

RESULTS

Physical interaction of IRF-1 and IRF-2 with TFIIB. To examine protein-protein interactions between TFIIB and members of the IRF family, agarose-based EMSA was performed with rIRF-1 produced from a baculovirus vector. Three copies of the GBP-ISRE (11) or the ISRE connected to a 40-bp basal promoter (containing a TATA box and initiator) (4) (GBP-ISRE-L^d40) was used as a probe (Fig. 1A). When rIRF-1 (10 ng) was added alone to a reaction, no detectable band was revealed by either probe (Fig. 1B, lanes 1 and 9) except when a large amount of IRF-1 (>80 ng) was added (data not shown). When TFIIB was added alone, no band was detected (lanes 7 and 15), which was expected, as TFIIB does not bind to double-stranded DNA (23). In contrast, when rIRF-1 and increasing amounts of TFIIB were added together, a strong band was detected in a TFIIB-dose-dependent manner with either probe (lanes 2 to 6 and 10 to 14). This binding was specific for the ISRE and did not involve the basal promoter, since (i) the binding was seen with the GBP-ISRE probe without basal elements and (ii) a competitor of the ISRE, but not one of the basal element, abrogated the band (Fig. 1B; compare lane 17 with lanes 18 and 19). The band contained both TFIIB and IRF-1, as antibodies against IRF-1 and TFIIB further shifted the band (Fig. 1B, lanes 20 and 21) but normal rabbit serum had no effect on the binding (lane 22). Agarose EMSA was also performed with in vitro-translated IRF-2 (Fig. 1C). When added alone, IRF-2 produced a weak, fast-migrating band (Fig. 1C, lane 2, black arrow). However, when IRF-2 was added together with TFIIB, a much stronger band was produced that migrated more slowly (Fig. 1C, lanes 3 and 4, open-headed arrow). The binding was again specific for the ISRE, as competitor oligomers of the ISRE, but not those of two control oligomers, eliminated the band (Fig. 1C, lanes 5 to 8). The band was further shifted by anti-TFIIB antibody and by anti-IRF-2 antibody but not by anti-IRF-1 antibody (lanes 9 to 12), confirming that the band represents the IRF-2–TFIIB complex. These results show that both IRF-1 and IRF-2 form a complex with TFIIB and bind to the ISRE with increased stability.

The DNA-binding domain of IRF-1 and the imperfect-repeat region of TFIIB are involved in the interaction. Interactions between IRF-1 and TFIIB were also investigated by using the GST-binding assay (Fig. 2A). ^{35}S-labeled full-length IRF-1 bound to GST-TFIIB but not to control GST (lanes 2 and 3), indicating specific binding of IRF-1 to TFIIB. In further support of the specificity of IRF-1 binding, several unrelated proteins tested as controls did not bind to GST-TFIIB (4) (data not shown). The GST-binding assay was also performed with IRF-1 constructs from which the C-terminal region of the parent protein was progressively deleted (Fig. 2A, lanes 4 to 12). ^{35}S-labeled IRF-1 peptides containing 295, 245, or 150 N-terminal amino acids all bound to GST-TFIIB, indicating that the N-terminal region corresponding to the DNA-binding domain is involved in binding to TFIIB. In agreement with these results, ^{35}S-labeled IRF-1 C-terminal domain (amino acids 121 to 329) did not bind to TFIIB. This binding was specific, since several control proteins did not bind to TFIIB (as presented in reference 4), and was not mediated by DNA, since inclusion of ethidium bromide did not affect the binding (33) (data not shown). To localize regions of TFIIB that interact with IRF-1, GST-TFIIB constructs containing various deletions (2, 24) were tested for binding to full-length IRF-1.

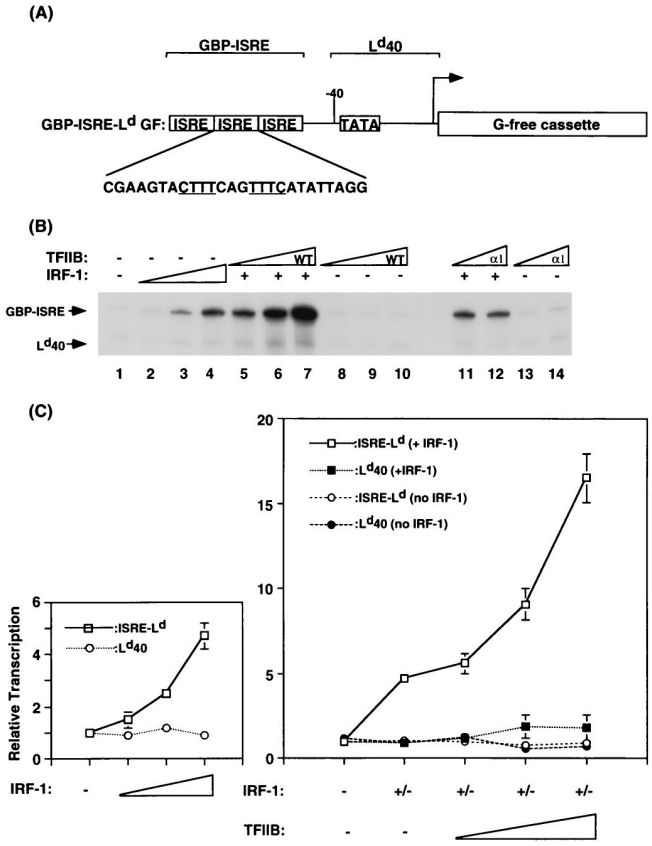

FIG. 4. rIRF-1 and rTFIIB cooperatively stimulate transcription from the ISRE promoter in vitro. (A) A schematic diagram of the GBP-ISRE-L^d40 G-free cassette. The promoter contained GBP-ISRE-L^d40 as shown in Fig. 1A. (B) In vitro transcription was carried out with the GBP-ISRE-L^d40 and L^d40 templates (200 ng each) in the presence of nuclear extracts from Namalwa cells (60 μg of protein). The reaction mixtures in lanes 2 to 4 contained 20, 40, and 80 ng of rIRF-1, respectively, while those in lanes 5 to 7 contained 80 ng of rIRF-1 plus 25, 50, and 100 ng of rTFIIB, respectively, and those in lanes 8 to 10 contained 25, 50, and 100 ng of rTFIIB, respectively. The reaction mixtures in lanes 11 and 12 contained 80 ng of rIRF-1 and 50 and 100 ng of the α1 mutant TFIIB, respectively, whereas the reaction mixtures in lanes 13 and 14 contained corresponding amounts of rTFIIB (50 and 100 ng, respectively) without rIRF-1. Transcripts are indicated by arrows. (C) Quantitation of in vitro transcripts. The relative levels of transcripts generated from the GBP-ISRE-L^d40 and the control (L^d40) templates were measured by densitometry analysis of the autoradiograms. The values represent the averages of three determinations ± the standard deviations.

As seen in Fig. 2B, removal of the N-terminal amino acids, including the zinc finger motif, did not affect IRF-1 binding. However, deletion of either of the imperfect repeats dramatically reduced IRF-1 binding (lanes 6 to 10). The TFIIB deletions were also tested for binding to IRF-1 in EMSAs (Fig. 2C). dN1 (4 to 24) and Δ1 (45 to 123), but not deletions in the C-terminal region, produced a retarded complex, which is in agreement with the GST binding data in Fig. 2B. These results indicate that the N-terminal region of IRF-1 and the C-terminal region of TFIIB are involved in the interaction.

Binding of endogenous cellular TFIIB to IRF-1. To further examine the interaction between IRF proteins and TFIIB, we performed a protein-binding assay on immobilized ISRE oligomers. A DNA fragment containing three copies of GBP-ISRE was immobilized onto magnetic beads and incubated with rIRF-1 and TFIIB, and bound proteins were detected by immunoblot assays. As shown in Fig. 3A, rIRF-1 bound to the ISRE beads in a dose-dependent fashion. This binding was specific, as free oligomers of the ISRE, but not those of pIRE, competed for binding (data not shown) and an unrelated recombinant protein, RXRβ or RARβ, did not bind to the beads (data not shown). As expected, when added alone, rTFIIB did not bind to the beads (Fig. 3B, lane 6) by itself. In the presence of rIRF-1, however, rTFIIB was retained on the ISRE beads in a dose-dependent manner (Fig. 3B, lanes 3 to 5). A rough quantitation of signals indicated that under these conditions about 25% of ISRE sites were occupied by rIRF (1 pmol per reaction), >85% of which bound to rTFIIB. These results confirm the physical interaction between TFIIB and IRF-1. This assay allowed us to assess interaction of the cellular TFIIB with rIRF-1. As shown in Fig. 3C, binding assays were performed with a fraction of nuclear extracts from Namalwa B cells (see below and Fig. 4). In immunoblot assays, cellular TFIIB was found mostly in a fraction separated by a P11 column and eluted with 0.5 M KCl (51). As shown in Fig. 3C, cellular TFIIB clearly bound to the ISRE beads with rIRF-1 (lane 3). It is of note that the cellular TFIIB migrated slightly faster than rTFIIB, as it lacked the histidine tag present in rTFIIB (lanes 5 and 6). Binding of cellular TFIIB was not detected when unfractionated nuclear extracts were used (data not shown). These results show that the endogenous TFIIB in nuclear extracts binds to IRF-1, further supporting the authenticity of the interaction.

rIRF-1 and rTFIIB cooperatively enhance transcription from an ISRE-containing template in vitro. To study the functional significance of the interaction described above, an in vitro transcription assay was developed. The template used in this assay contained three copies of the GBP-ISRE and a 40-bp basal promoter identical to GBP-ISRE-L^d40 (Fig. 1), which was fused to a G-free cassette (Fig. 4A). The control template, L^d40, consisted of only the basal promoter fused to a shorter G-free cassette (34). These templates were mixed with purified rTFIIB, rIRF-1, and nuclear extracts from Namalwa B cells as a source of other basal factors and RNA polymerase II. As seen in lanes 1 to 4 of Fig. 4B, in the absence of rIRF-1, the levels of transcripts produced from the ISRE template were very low, but addition of rIRF-1 gave a dose-dependent increase in transcript levels. Addition of rIRF-1, however, had no effect on the levels of transcripts produced by the control L^d40 template. Thus, IRF-1 acted as an activator of the ISRE promoter in vitro, consistent with previous results in vivo (25, 45, 49, 52). As seen in lanes 5 to 7, addition of increasing amounts of rTFIIB in the presence of rIRF-1 led to a strong, dose-dependent increase in transcription from the ISRE template with a very minor increase in transcription from the control template (see quantitation in Fig. 4C). In the absence of rIRF-1, rTFIIB had no effect on transcription from either the ISRE or the control template (lanes 8 to 10). In addition, rIRF-1 and TFIIB did not significantly increase the levels of transcription from the adeno major late promoter, another control template without ISRE (data not shown). As seen in lanes 11 to 14, a mutant TFIIB that failed to interact with IRF-1 (Fig. 2B and C) also failed to increase transcription by rIRF-1 from the ISRE template. The enhanced transcription was reproducibly observed with different preparations of rIRF-1, rTFIIB, and nuclear extracts (data not shown). These results show that rIRF-1 and rTFIIB enhance transcription from the ISRE template in vitro, supporting the physical interaction described above.

TFIIB exerts positive and negative effects on ISRE promoter activity in a cell type-dependent manner. Functional interaction between TFIIB and IRF-1 was further investigated in vivo by transient transfection assays using a luciferase reporter gene

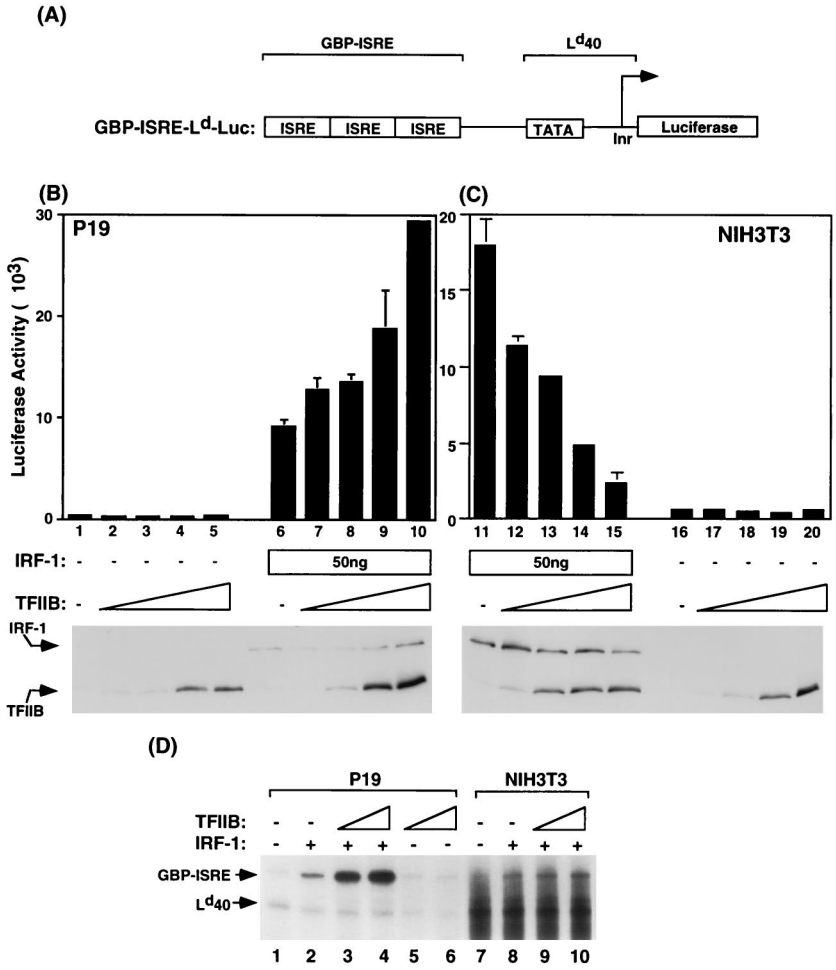

FIG. 5. Dual effects of TFIIB on ISRE reporter activity. (A) A schematic diagram of the reporter construct. The promoter is the same as GBP-ISRE-L^d40 used in the experiment detailed in Fig. 1 but is fused to the luciferase gene. (B) Cooperative enhancement of ISRE promoter activity by IRF-1 and TFIIB in P19 EC cells. P19 cells were transfected with 0, 200, 400, 800, or 1,600 ng of pRSV-hTFIIB (lanes 1 to 5 and 6 to 10, respectively) in the absence or presence of pAct-1 (IRF-1, 50 ng) and the ISRE luciferase reporter gene (400 ng). The values represent the averages of the data from quadruplicate assays ± the standard deviations. The lower panel is the corresponding immunoblot showing the levels of IRF-1 and TFIIB expressed in the transfected cells. (C) Repression of ISRE promoter activation by TFIIB in NIH 3T3 cells. Transfection was carried out with the Lipofectamine procedure. The values represent the averages of the data from quadruplicate assays ± the standard deviations. The lower panel is the corresponding immunoblot showing the levels of IRF-1 and TFIIB expressed in the transfected cells. (D) In vitro transcription from the ISRE-L^d40 template with nuclear extracts (60 μg of protein) from P19 or NIH 3T3 cells. In vitro transcription was carried out as described in the legend to Fig. 4. The reaction mixtures in lanes 2 to 4 and 8 to 10 contained 80 ng of rIRF-1, while those in lanes 3 and 4 and in lanes 9 and 10 contained 50 and 100 ng of rTFIIB, respectively.

fused to the GBP-ISRE-L^d40 promoter, identical to that used for in vitro transcription (Fig. 4A and 5A). P19 EC cells were chosen for transfection assays, since IRF proteins are not expressed in these cells (26). As seen in Fig. 5B, in the absence of IRF-1, the ISRE reporter gave a background activity with and without cotransfected TFIIB (lanes 1 to 5), as expected. However, cotransfection of the IRF-1 vector led to a ~30-fold increase in luciferase activity in the absence of TFIIB (lane 6), in agreement with previous reports (26, 49). Furthermore, when IRF-1 and increasing amounts of TFIIB were cotransfected, luciferase activity was further increased in a dose-dependent fashion, reaching a maximum increase in reporter activity of ~90-fold (lanes 7 to 10). Luciferase activity of the control reporter fused to the L^d40 basal promoter was not significantly increased by transfection of IRF-1 or TFIIB, as noted before (4, 49). The immunoblot analyses whose results are shown in Fig. 5B and C confirmed that transfected IRF-1 and TFIIB were appropriately expressed in these cells. These results show that TFIIB and IRF-1 cooperatively enhance ISRE promoter activity in P19 EC cells, demonstrating a functional interaction of the two factors in vivo. Similar cooperative promoter stimulation was observed with TFIIB and another sequence-specific transcription factor, vitamin D receptor, in P19 EC cells (4). In that study we noted that in another cell type, i.e., NIH 3T3 cells, cotransfection of TFIIB repressed promoter activity, suggesting a dual activity of TFIIB that depends on cell type. To further investigate the effects of TFIIB on ISRE promoter activity in vivo, cotransfection assays were performed with NIH 3T3 cells; the results are presented in Fig. 5C. Basal reporter activity in the absence of transfected IRF-1 was slightly higher in NIH 3T3 cells than in P19 EC cells, presumably reflecting the activity of the endogenous IRF-1 (lane 16). As in P19 EC cells, transfection of TFIIB alone did not affect reporter activity (lanes 17 to 20). Transfection of IRF-1 alone increased luciferase activity by >20-fold (lane 11). However, cotransfection of increasing amounts of TFIIB resulted in a dose-dependent reduction in luciferase activity (lanes 12 to 15). These results indicate that TFIIB

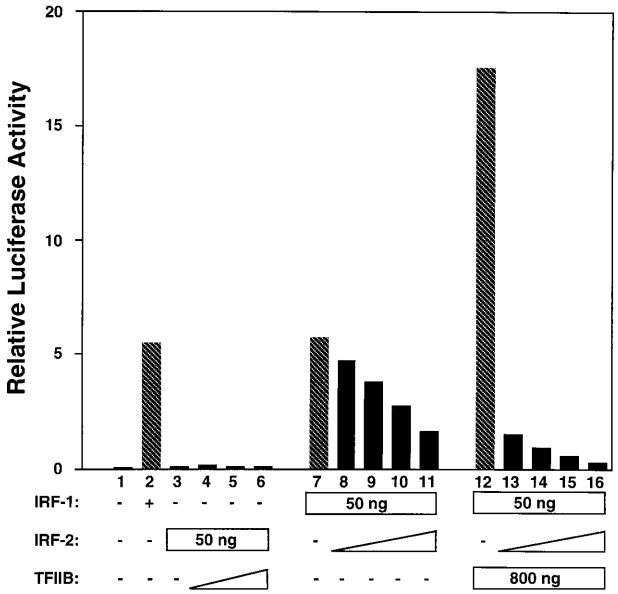

FIG. 6. IRF-2 and TFIIB synergistically inhibit ISRE promoter activation. P19 cells were transfected with 50 ng of pAct-1 (IRF-1) (lane 2) or pAct-2 (IRF-2) (lanes 3 to 6) and increasing amounts of pRSV-hTFIIB (200, 400, or 800 ng). In lanes 7 to 16, the IRF-1 plasmid (50 ng) and increasing amounts of the IRF-2 plasmid (6, 12, 25, and 50 ng) were transfected with or without the TFIIB plasmid (800 ng). The hatched bar represents a control transfection with the IRF-1 plasmid but not the IRF-2 plasmid. The values represent the averages of the data from three experiments.

represses IRF-1-mediated promoter activation in NIH 3T3 cells. The contrasting effects of exogenous TFIIB seen in P19 EC and NIH 3T3 cells were not attributed to the levels of endogenous TFIIB expressed in these cells, since immunoblot assays showed that the two cells express comparable levels of TFIIB (4).

The cell type-dependent effects of TFIIB were further explored by in vitro transcription. As shown in Fig. 5D, nuclear extracts prepared from P19 EC cells or NIH 3T3 cells were tested for their ability to drive transcription from the ISRE template in the presence of rIRF-1 and rTFIIB. With extracts from P19 cells, addition of rIRF-1 and rTFIIB led to enhanced levels of transcription from the ISRE template in a dose-dependent manner, without enhancing transcription from the control L^d40 basal promoter (Fig. 5D, lanes 3 and 4). Similar to the results in Fig. 4, addition of rTFIIB alone, without rIRF-1, did not enhance transcription (lanes 5 and 6). Extracts from NIH 3T3 cells, however, failed to give enhanced transcription; although addition of rIRF-1 resulted in a specific increase in transcription from the ISRE template, further addition of rTFIIB did not increase the level of transcription (lanes 9 to 10). Taken together, these results support the view that the functional interaction between TFIIB and IRF-1 is modulated by another factor that is expressed (or functional) in a cell type-specific fashion.

IRF-2 and TFIIB synergistically inhibit ISRE promoter activation. IRF-2 counteracts IRF-1 and represses ISRE promoter activity stimulated by IRF-1 or by IFN (25, 49). Since IRF-2 also interacted with TFIIB and bound to ISRE (Fig. 1C), it was of importance to study whether the repressive function of IRF-2 is affected by TFIIB. Figure 6 shows the results of transfection assays performed in P19 EC cells. Transfection of IRF-2 alone resulted in a modest increase in luciferase activity from the ISRE promoter, albeit to a much lesser degree than transfection of IRF-1 (compare lanes 2 and 3). The enhancement of promoter activity by IRF-2 may be due to a cryptic activation domain present in IRF-2 (68). When IRF-1 was transfected with increasing amounts of IRF-2 in the absence of TFIIB, ISRE reporter activity was repressed in a dose-dependent manner (lanes 8 to 11), confirming the repressive role of IRF-2. Furthermore, when IRF-1 and IRF-2 were cotransfected with TFIIB, luciferase activities were greatly reduced (lanes 13 to 16), and the extent of reduction was dependent on the dose of IRF-2. In the presence of TFIIB, even the smallest amount of IRF-2 (6 ng) led to a >90% inhibition of luciferase activity (compare lanes 12 and 13), while in the absence of TFIIB, there was only ~10% inhibition (compare lanes 7 and 8). With the largest amount of IRF-2 (50 ng), the luciferase activity was almost completely inhibited in the presence of TFIIB but not in its absence. These results indicate that IRF-2 and TFIIB synergistically repress IRF-1 activation of the ISRE promoter.

The role of the TATA and Inr sequences in ISRE reporter activity: cell type-specific TATA requirement. Although earlier studies indicated that the TATA box is essential for transcription initiation, more recent studies have shown that Inr directs transcription from TATA-less promoters (29, 58). Inr has been shown to play a role, even for those promoters that contain a TATA box, by increasing promoter strength (8). In this work, we have addressed the role of a TATA box in ISRE promoter activity. Depicted in Fig. 7A are the results of experiments in which TATA mutations were placed in the GBP-ISRE-L^d40 luciferase reporters (mt2 to mt4). Promoter activity of these mutants was tested following transfection of IRF-1 and TFIIB into P19 EC cells (Fig. 7A) and NIH 3T3 cells (Fig. 7B). In P19 EC cells, all three mutant promoters were activated by IRF-1. Furthermore, all the mutants demonstrated cooperatively enhanced promoter activity upon TFIIB cotransfection in a dose-dependent fashion (lanes 7 to 21). Levels of activation by these mutants were comparable to those seen with the wild-type reporter with the intact TATA box (lanes 2 to 6). In contrast, the mt2 and mt4 promoters failed to enhance luciferase activity in NIH 3T3 cells upon transfection of IRF-1, and cotransfection of TFIIB led to only a minor reduction in luciferase activity (Fig. 7B). The mt3 construct, which contained a conservative mutation, behaved like the wild-type promoter, as reported previously (3). These results show that ISRE reporter activation by IRF-1 and cooperative enhancement by TFIIB do not require the TATA box in P19 EC cells, while it is required in NIH 3T3 cells.

We examined the role of the Inr in P19 EC cells in ISRE reporter activity by using a mutant construct in which the Inr sequence in the GBP-ISRE-L^d40 luciferase was extensively altered (Inr-mt) (Fig. 7C). The wild-type reporter was stimulated by IRF-1 and further augmented by TFIIB, as expected (lanes 2 to 5). However, stimulation of the Inr-mt by IRF-1 was only about 50% of that of the control (lane 7), and cotransfection of TFIIB did not give any further increase in reporter activity (lanes 8 to 10). Another mutant in which both the Inr and TATA sequences were mutated gave essentially the same pattern of response (lane 11 to 15) as Inr-mt, confirming the dispensability of the TATA sequence. These results indicate the critical role played by the Inr in ISRE promoter activation by IRF-1 and TFIIB in P19 EC cells.

Modulation of IRF-1–TFIIB interaction during RA-induced differentiation of P19 cells. To gain insight into the basis of the cell type-specific activity of TFIIB described above, transfection assays were performed with P19 cells induced to differentiate by RA treatment. Treatment with RA (10^{-6} M) for 2 weeks resulted in distinct morphological alterations which

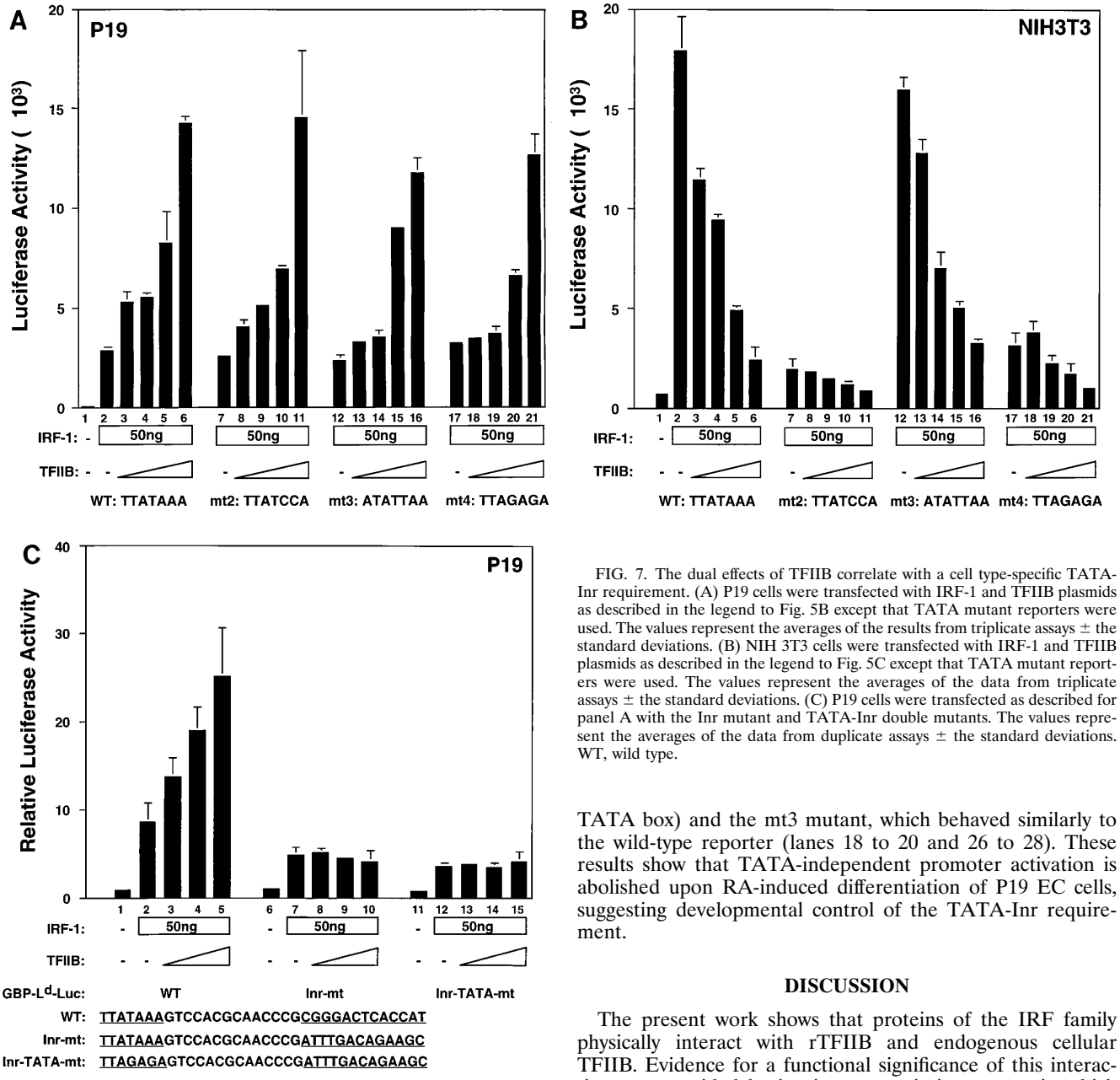

FIG. 7. The dual effects of TFIIB correlate with a cell type-specific TATA-Inr requirement. (A) P19 cells were transfected with IRF-1 and TFIIB plasmids as described in the legend to Fig. 5B except that TATA mutant reporters were used. The values represent the averages of the results from triplicate assays ± the standard deviations. (B) NIH 3T3 cells were transfected with IRF-1 and TFIIB plasmids as described in the legend to Fig. 5C except that TATA mutant reporters were used. The values represent the averages of the data from triplicate assays ± the standard deviations. (C) P19 cells were transfected as described for panel A with the Inr mutant and TATA-Inr double mutants. The values represent the averages of the data from duplicate assays ± the standard deviations. WT, wild type.

were characteristic of differentiation. As seen in Fig. 8, ISRE promoter activity in the absence of transfected IRF-1 was slightly higher in RA-treated P19 cells than in untreated P19 EC cells (lanes 1 and 17), presumably reflecting RA-induced expression of IRFs (26). Nevertheless, transfection of IRF-1 resulted in strong ISRE promoter activation, similar to that seen in untreated P19 cells (lane 18). However, cotransfection of TFIIB did not significantly increase luciferase activity (lanes 19 and 20) in RA-treated cells, in contrast to the strong cooperative activation seen in untreated P19 cells (compare with lanes 3 and 4). Furthermore, transfection of IRF-1 did not efficiently activate the mt2 and mt4 TATA mutants in RA-treated P19 cells, either in the presence or absence of TFIIB (lanes 22 to 24 and 30 to 32); levels of activation were less than 50% of those of the control reporter (containing the wild-type TATA box) and the mt3 mutant, which behaved similarly to the wild-type reporter (lanes 18 to 20 and 26 to 28). These results show that TATA-independent promoter activation is abolished upon RA-induced differentiation of P19 EC cells, suggesting developmental control of the TATA-Inr requirement.

DISCUSSION

The present work shows that proteins of the IRF family physically interact with rTFIIB and endogenous cellular TFIIB. Evidence for a functional significance of this interaction was provided by in vitro transcription assays, in which rIRF-1 and rTFIIB caused cooperative enhancement of transcription from an ISRE promoter. Furthermore, in vivo transfection assays revealed that TFIIB affects ISRE promoter activity positively or negatively depending on the cell type, which correlates with the requirement of TATA and Inr for promoter activation. The cell type-dependent activity of TFIIB was also noted during in vitro transcription with extracts from different cells and was found to be dependent on differentiation of EC cells. Our results indicate that IRF proteins and TFIIB interact with each other in concert with a cell type-specific factor.

Physical interaction. We found that both IRF-1 and IRF-2 directly interact with TFIIB (Fig. 1 and 2) and that this interaction facilitates their binding to the ISRE. TFIIB-facilitated DNA binding has also been reported for the POU domain-containing Oct-1 (47). In a similar context, association of P53 with a GTF (TBP) has been shown to strengthen DNA binding (6).

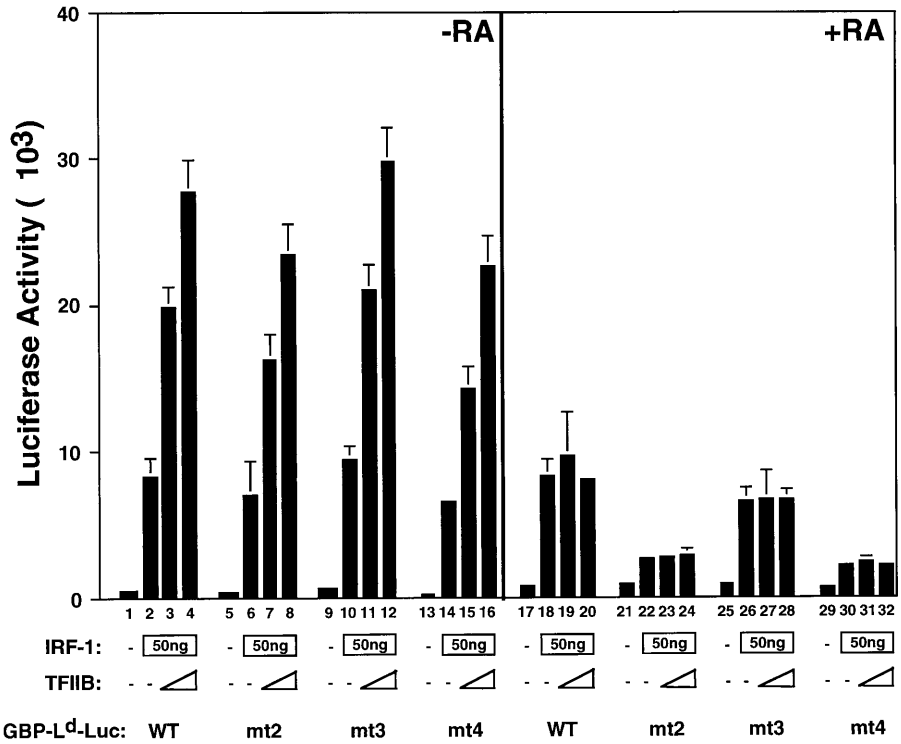

FIG. 8. Loss of the positive effect of TFIIB and TATA independence in RA-treated P19 cells. P19 cells were cultured in the absence (−RA) or presence (+RA) of all-*trans* RA (10^{-6} M) for 2 weeks prior to transfection. Cells were transfected with the IRF-1 plasmid (50 ng) and increasing amounts of the TFIIB plasmid (800 to 1,600 ng). The values represent the averages of data from duplicate assays ± the standard deviations. WT, wild type.

IRFs interact with other transcription factors, including ICSBP, another member of the IRF family (5); NF-κB (13, 48); and HMG(Y) (48). Some of these interactions also affect the ability of IRFs to bind to the ISRE (5, 48). Thus, the binding of a TFIIB-IRF complex to the ISRE may also be regulated by other transcription factors. Deletion analysis (Fig. 2) indicated that the interaction involves the N-terminal domain of IRF-1, suggesting that this domain is involved in DNA-binding activity and protein-protein interactions. The N-terminal domains of IRF-1 and IRF-2 have also been shown to be essential for interaction with NF-κB proteins (13). It is possible that this domain undergoes a conformational change upon interaction with other proteins, leading to enhanced binding to the ISRE.

Immobilized-bead assays (Fig. 3) showed that the endogenous TFIIB in B cells interacts with rIRF-1, lending credence to the IRF-TFIIB interaction seen with rTFIIB. The relatively weak binding noted with the endogenous TFIIB is likely to be due to the low TFIIB concentrations present in the extracts (estimated to be <10 ng, relative to 100 ng of rTFIIB per reaction tested in these assays). Some of the TFIIB in this fraction may be associated with other factors and may even be part of the RNA polymerase II holoenzyme complex (50). Studies to analyze the molecular nature of the endogenous TFIIB bound to IRF-1 are under way.

Functional interaction. In the present study we have established an in vitro assay in which transcription from an ISRE-containing template was specifically activated by rIRF-1. Previously, Fu et al. (18) reported in vitro transcription with natural ISGF3 preparations obtained from HeLa cells. However, to our knowledge, this is the first report of successful in vitro transcription from the ISRE promoter with a recombinant IRF protein. We also showed that rIRF-1 and rTFIIB synergistically enhance transcription from the ISRE template without significantly affecting levels of basal transcription (Fig. 4), supporting the significance of the physical interactions noted above. These results indicate that rIRF-1, by complexing with rTFIIB, is assembled into a functional transcription machine that drives ISRE-specific transcription in vitro. The increased transcription may be attributed not only to increased IRF-1 binding to the ISRE but also to enhanced formation of

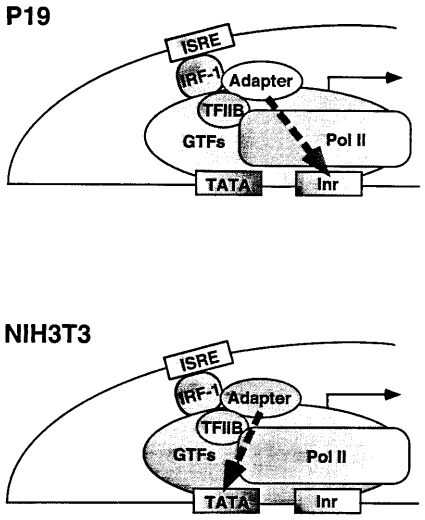

FIG. 9. A model for IRF-TFIIB interaction. A cell type-specific factor (adapter) is postulated that mediates TFIIB-IRF interactions and drives TATA- or Inr-dependent transcription in vivo.

the PIC. In accordance with these results, Choy and Green (7) have shown that the interaction of TFIIB with acidic and nonacidic activators increases PIC assembly. In addition, it has been shown that TFIIB decreases the dissociation rate of COUP-TF, thus increasing transcription (61). More recent work (53) indicates that binding of VP16 alters the conformation of TFIIB, leading to enhanced recruitment of basal factors and transcription initiation. Considering that VP16 binds to a region of TFIIB very similar to that used for IRF binding, a similar change in TFIIB conformation may be expected following binding to IRFs.

In this work, efforts have been made to study the nature of the functional interaction between TFIIB and IRFs in vivo. We have shown that transfection of TFIIB causes opposite effects on ISRE promoter activity in P19 EC and NIH 3T3 cells. The positive and negative effects seen in the two cell types were not attributable to different levels of TFIIB, since TFIIB levels in these cells were found to be comparable (4) (Fig. 5B and C). Consistent with the in vivo data, addition of rTFIIB enhanced ISRE promoter transcription in the presence of P19 cell extracts but not with NIH 3T3 cell extracts (Fig. 5), supporting the involvement of a cell type-specific factor(s). It is likely that TFIIB acts in concert with another factor, such as an adapter, that modulates the interaction with IRFs in vivo in a cell type-specific manner (see the model in Fig. 9). It is noteworthy that transfection of TFIIB produced similar positive and negative effects on ligand-mediated transcription by vitamin D receptor (4), indicating that the function of such an adapter is not restricted to proteins of the IRF family and may be seen broadly with other activators.

Interestingly, the cell type-specific positive and negative activities of TFIIB correlated with the TATA requirement of ISRE promoter activity (Fig. 7). While in P19 EC cells mutations in the TATA sequence had no inhibitory effect on reporter activation either in the presence or absence of transfected TFIIB, these mutations abrogated promoter activation in NIH 3T3 cells and reduced the inhibitory effect of TFIIB. It is possible that the cell type-specific factor that acts on the TFIIB-IRF interaction is involved in directing the TATA requirement for transcription (Fig. 9). In P19 EC cells the Inr, rather than a TATA sequence, appears to play a major role, as a mutation in the Inr significantly reduced ISRE promoter activation by IRF-1 and TFIIB (Fig. 7C). Inr-dependent but TATA-independent transcription has been previously noted in P19 EC cells with the RARβ2 promoter (12). The Inr has been shown to direct basal-factor assembly and transcription initiation in a number of TATA-less promoters (58) (for a review, see reference 57). The Inr even functions in TATA-containing promoters and increases the strength of transcription initiation (8, 58). Transcription initiation via the Inr appears to involve TBP and TFIID, since the Inrs of both TATA-containing and TATA-less promoters have been shown to bind to TFIID (30, 66). Furthermore, the binding of TFIID to the Inr is reported to be strongly affected by TFIIB (30), raising the possibility that TFIIB has a greater role in promoter activation mediated through an Inr than in that mediated through a TATA sequence (Fig. 9).

At present, the factor(s) that affects the IRF-TFIIB interaction and the mechanisms of its action are not known. Because TFIID is heterogeneous and is composed of different TAFs (28), and because TFIIB is capable of interacting with at least one TAF (20), it is possible that TAFs play a role. However, other factors known to interact with Inr, such as YY1, E2F, and upstream stimulatory factor (16, 44, 56, 62), may have a significant role.

By studying P19 cells treated with RA we found that the positive effect of TFIIB as well as TATA-independent promoter activation are both abolished upon differentiation of cells. Our results indicate that cell type specificity is not an arbitrary variability among various cultured cells but reflects the developmental state of the cells. In agreement, Majumder and DePamphilis (41) showed that a TATA box is not required for enhancer stimulation in early mouse embryos but is required in differentiated fibroblasts and oocytes. Thus, the assembly and function of the RNA polymerase II-dependent initiation complex may be developmentally controlled. It would be of importance to identify a factor(s) that mediates the TFIIB-IRF interaction and to study its developmental role in promoter activation.

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