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Purification and Properties of the Rous Sarcoma Virus Internal Enhancer Binding Factor

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The internal enhancer binding factor (IBF) that specifically binds sequences within the *gag* gene internal enhancer of Rous sarcoma virus Schmidt-Ruppin A was purified to near homogeneity from BHK cells. The polypeptides that constituted IBF DNA-binding activity were identified by sodium dodecyl sulfate-polyacrylamide gel analysis. As isolated from BHK cells, IBF consisted of two different but related polypeptides. One (IBFα) had a molecular weight of 40,000; the other (IBFβ) had a molecular weight of 20,000 and appeared to be a proteolytic product of IBFα. The site within the *gag* gene to which IBF bounds in vitro (internal enhancer site 2; nucleotides 856 to 878 of the Rous sarcoma virus genome) were demonstrated to function as a *cis*-acting transcriptional stimulatory element both in vivo and in vitro. By using HeLa cell nuclear transcription extracts, purified IBF was found to function as a *trans*-acting transcription factor that stimulated transcription in vitro. Purified IBF was also demonstrated to be very similar to EBP20 (K. Carlberg, T. A. Ryden, and K. Beemon, J. Virol. 62:1617–1624, 1988), and it may well belong to the same family of DNA-binding proteins.

Rous sarcoma virus (RSV) has been used as a model system for identifying enhancers and their functions and for identifying viral oncogenes (13, 34). The sequences required for transcriptional enhancement, initiation, and termination are located within the long terminal repeats (10, 11, 18, 31, 32). Recently, the *gag* coding sequences have been identified as possibly playing a role in control of the proviral life cycle; they may also play a role in the ability of the virus to transform cells both in vitro (47) and in vivo (42). A transcriptional enhancer has been identified within this region (1, 29, 47), and the *cis*-acting enhancer sequences may be responsible for the transformation effects observed in this region. When assayed by transient transfection, this internal enhancer increases transcription about fivefold less efficiently than does the enhancer located within the long terminal repeat. However, the internal enhancer can act synergistically with the long-terminal-repeat enhancer to further increase long-terminal-repeat-driven transcription (1). These data, therefore, suggest that the internal enhancer may play a crucial role in the transcriptional control of this virus, although it is not yet known whether the sequences identified in this report and by others (6) are important in the viral life cycle. The internal enhancer has also been shown to function in a wide variety of cell types. Moreover, infection of cells with the intact virus is not required for internal enhancer function (1, 29, 47). These observations indicate that these *cis*-acting sequences bind cellular transcription factors that are highly conserved.

Enhancers have the property of activating transcription from promoters in a distance- and orientation-independent manner (2, 3, 16). Enhancers are composed of short modular sequence elements which by themselves have no enhancer function unless the elements are ?present in multiple copies or other enhancer elements are also present (22, 23, 51). Recently, enhancer elements have been demonstrated to bind specific nuclear proteins (4, 36, 37, 44, 50); in some cases, these proteins have been purified (8, 25, 26, 28, 48) and the genes encoding them have been cloned (27, 46).

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In this report, we further characterize these RSV DNA sequences (internal enhancer site 2 [IES2]; 29) that were previously documented to interact with BHK cell nuclear proteins. We show that RSV IES2 (nucleotides 856 to 878) constitutes a *cis*-acting element which stimulates transcription both in vitro and in vivo. Moreover, the BHK nuclear DNA-binding protein that interacts with this sequence element was purified over 700,000-fold to near homogeneity, with a very good yield of activity. Some of the properties of this protein, termed internal enhancer binding factor (IBF), have been characterized.

Intact IBF appears to be a dimer composed of two identical subunits (α) with a subunit molecular weight of 40,000. During the preparation procedure, many of the intact 40,000-molecular-weight α subunits are proteolyzed into 20,000-molecular-weight β subunits. The 40,000- and 20,000-molecular-weight subunits have been termed IBFα and IBFβ, respectively. Thus, the majority of IBF in a nuclear extract is in the form IBFαβ. In principle, however, three different dimers may be formed: IBFαα, IBFαβ, and IBFββ. Each dimer yields a distinct protein-DNA complex that can be resolved from the others when assayed by the DNA mobility shift technique. DNA binding to IBFαα, IBFαβ, or IBFββ yields what we have termed complex III, complex II, and complex I, respectively. The ability of IBF to function as a transcription factor was also tested. Purified IBF specifically stimulated mammalian extract-mediated RNA polymerase II transcription in vitro in an IES2 sequence-dependent fashion.

The relationship of IBF to other transcription factors is not yet known, although Carlberg et al. (6) have recently reported that the rat liver protein EBP20 (25) binds to the RSV internal enhancer. IBF, isolated from fibroblasts, appears to be very similar to EBP20 and may belong to the same family of DNA-binding proteins.

MATERIALS AND METHODS

Plasmid construction. To generate multimers of the IBF binding site, the duplex oligonucleotide

CAGCTGTTGGCTGCAATTGCGCCACCGCCACAG
GACAACCGACGTTAACGCGGTGGCGGTGTCGTC

was prepared from its gel-purified single strands and treated with T4 DNA ligase (New England BioLabs, Inc., Beverly, Mass.). The mixture of ligated oligomers was treated with the Klenow fragment to produce blunt ends. The entire ligation mixture was blunt-end ligated into the *Eco*RV site of the Bluescript vector (Stratagene). The resulting recombinants were then screened by the alkaline lysis procedure (33), and those containing one, two, three, or eight multimers of the oligonucleotide were selected. To subclone these multimers into the OVEC vectors (49), the plasmids containing one, three, or eight inserts were digested with *Hin*dIII and *Eco*RI and converted into blunt ends by treatment with the Klenow fragment. The blunt-ended restriction fragments were then electrophoresed on a 10% polyacrylamide gel, and the multimer-containing restriction fragments were electroeluted and subcloned directly into the *Sal*I site of OVEC1, which had been converted to a blunt end by use of the Klenow fragment. This procedure generated three plasmids, designated pO^1VEC, pO^3VEC, and pO^8VEC, which contained one, three, and eight copies, respectively, of the duplex oligonucleotide sequence shown above. All plasmids were propagated in *Escherichia coli* DH5α.

Assay methods. DNA mobility shift assays were carried out as previously described (15, 29). All mobility shift assays were performed with the ^{32}P-labeled oligonucleotide OpvuII3′. The sequence of OpvuII3′ is

CTGTTGGCTGCAATTGCGCCACCGCCACAG
GACAACCGACGTTAACGCGGTGGCGGTGTC

The nonspecific competitor used for DNA mobility shift competitions was a *ras* nuclear factor I-binding site (26), the sequence of which is

TCGAATGGCGCGCAGCCAATGGTAGGCC
AGCTTACCGCGCGTCGGTTACCATCCGG

Transfection experiments were performed as previously described (19). BHK cells used for the preparation of nuclear extracts and transfections were grown in Dulbecco modified Eagle medium supplemented with 2.5% (vol/vol) bovine serum–7.5% (vol/vol) fetal calf serum or in 10% (vol/vol) iron-supplemented calf serum (Hyclone). Sodium dodecyl sulfate (SDS)-polyacrylamide gels were run according to the method of Laemmli (30). Protein standards (Sigma Chemical Co., St. Louis, Mo.) used as molecular size markers were carbonic anhydrase (29 kilodaltons [kDa]), ovalbumin (43 kDa), bovine serum albumin (68 kDa), phosphorylase *b* (97 kDa), β-galactosidase (116 kDa), and myosin (205 kDa). Protein concentrations were assayed by using the Bradford reagent (5; Bio-Rad Laboratories, Richmond, Calif.), with bovine serum albumin as the standard.

Purification of IBF. IBF DNA-binding activity was monitored during the purification procedures by the gel shift assay. All steps were carried out at 4°C unless otherwise stated. Nuclear extracts were isolated from 15 to 20 g of BHK cells as previously described (29, 43) except that inhibitors were present in the isolation and extraction buffers at the following concentrations: 20 mM benzamidine, 2 mM phenylmethylsulfonyl fluoride (PMSF), 20 mM β-glycerophosphate, 10 μg of leupeptin per ml, and 1.4 μg of pepstatin per ml. The 0.5 M NaCl extraction was replaced by 0.8 M extraction. Briefly, the nuclei were prepared by Triton X-100 lysis and then washed with 5 volumes of nuclear extraction buffer containing 0.3 M NaCl. IBF was then extracted from the nuclei by incubation of the nuclei in 4 volumes of nuclear extraction buffer plus 1 M NaCl (final NaCl concentration, approximately 0.8 M) for 1 h with occasional mixing. The resulting viscous suspension was then centrifuged for 1 h at 82,000 × *g*. After centrifugation, the supernatant was removed and diluted to 0.28 M NaCl with buffer A (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 8.0], 5% [vol/vol] glycerol, 1 mM EDTA, 1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], 0.1% [vol/vol] Nonidet P-40, 7 mM β-mercaptoethanol, 10 mM benzamidine, 1 mM PMSF, 1.7 μg of pepstatin per ml, 5 μg of leupeptin per ml) containing 10 mM β-glycerophosphate. The resultant precipitate was removed by centrifugation at 16,000 × *g* for 15 min, and the supernatant was applied to a heparin-Sepharose column at a ratio of 5 mg of protein per ml of packed resin. The heparin-Sepharose column was prepared as previously described (12). The column was then extensively washed with buffer A containing 0.3 M NaCl until no protein eluted. IBFαβ and IBFββ were eluted with buffer A containing 0.7 M NaCl and 1 mM β-glycerophosphate. The protein peak fractions were pooled and applied to a hydroxylapatite (Bio-Rad) column at a ratio of 4 mg of protein per ml of resin. The fold purification and the phosphate concentrations required to separate IBFαβ and IBFββ varied from lot to lot of hydroxylapatite and were empirically determined. The column was washed with the 0.7 M application buffer, and IBFββ was eluted with buffer A containing 2 M NaCl and 10 mM β-glycerophosphate. IBFαβ was then eluted with 2 M NaCl–40 mM sodium phosphate (pH 8.0)–5% (vol/vol) glycerol–0.1 mM EDTA–7 mM β-mercaptoethanol, 0.01% (vol/vol) Nonidet P-40–10 mM benzamidine–10 mM β-glycerophosphate–1 mM PMSF–1.7 μg of pepstatin per ml–5 μg of leupeptin per ml. The proteins in the peak were pooled and dialyzed into the same buffer without sodium phosphate and containing 50 mM NaCl and 10 mM HEPES (pH 8.0). The dialysis was stopped when the NaCl concentration was approximately 150 mM. The dialyzed material was then diluted twofold with dialysis buffer, placed in a boiling water bath for 5 min, and cooled on ice for 15 min. The insoluble material was removed by centrifugation at 16,000 × *g* for 10 min. To the supernatant was added 0.05 μg of freshly boiled, sheared salmon testes DNA per μg of protein, along with poly(dI-dC) · poly(dI-dC) (0.3 μg/μg of protein), wheat germ tRNA (0.8 μg/μg of protein), and spermidine to a final concentration of 5 mM. This protein-nucleic acid mixture was then applied batchwise to a DNA affinity column at a ratio of 0.3 mg of protein per ml of resin and incubated on a rotating platform for 4 h. The DNA affinity column was prepared as previously described (28). The oligonucleotide sequence used to construct the OVEC vectors was also used to prepare the DNA affinity column. Ligated oligonucleotides (average of 10-mers) were coupled to the resin for a final yield of approximately 40 μg of DNA per ml of resin. After loading, the column was unplugged, the binding mix was allowed to run through, and the column was washed with buffer O (10 mM HEPES [pH 8.0], 5% glycerol, 0.1 mM EDTA, 7 mM β-mercaptoethanol, 0.05 mg of insulin per ml, 1 mM benzamidine, 0.1 mM PMSF, 1 mM β-glycerophosphate, 0.01% [vol/vol] Nonidet P-40, 5 μg of leupeptin per ml, 0.7 μg of pepstatin per ml) containing 150 mM NaCl. IBFαβ was then eluted with buffer O containing 0.7 M NaCl. The activity peak fractions were pooled and diluted to 175 mM NaCl with buffer O; then poly(dI-dC) · poly(dI-dC) was added to 0.05 mg/ml, and the pooled fractions were applied to a DNA-Sepharose column one-fifth the volume of the

original column and allowed to bind for 2 h on a rotary platform at 4°C. The column was then developed exactly as described above for the first pass over the DNA-Sepharose. The activity peak fractions were pooled and frozen at −70°C.

Transcription extract preparation. HeLa cells were grown in spinner flasks in Joklik modified Eagle media supplemented with 2.5% (vol/vol) fetal bovine serum, 2.5 % (vol/vol) calf serum, 1 mM glutamine, and 1 mM nonessential amino acids. Cells were harvested during the log phase of growth at a density of 3×10^5 to 4×10^5 cells per ml. Usually 3×10^9 cells was used for a nuclear extract preparation. Transcription extracts were prepared by the method of Shapiro et al. (45). Nuclear extracts typically contained 20 to 22 mg of protein per ml after dialysis, and they were frozen quickly in small portions on dry ice and stored at −70°C.

In vitro transcription reactions. Transcription reactions contained 40 to 300 μg of HeLa nuclear extract. Reactions were performed in 8 mM HEPES (pH 7.9)–8% (vol/vol) glycerol–70 to 80 mM KCl–80 μM EDTA–80 μM EGTA–80 μM dithiothreitol–7 mM $MgCl_2$–1 mg of bovine serum albumin (Miles) per ml–500 μM each ATP, UTP, GTP, and CTP in a final volume of 50 μl. Purified IBFαβ or the appropriate buffer was added to the template DNA 15 min before addition of transcription extract and incubated at 30°C. The transcription reaction was initiated by addition of the transcription extract. Transcription reactions were incubated at 30°C for 60 min and stopped by addition of 50 μl of a solution containing 1 mg of *E. coli* tRNA per ml, 0.4% (wt/vol) SDS, 100 mM sodium acetate (pH 5.5), and 1,000 cpm of ^{32}P-labeled DNA (double-stranded tracer DNA added as an internal recovery control standard) per assay. Transcription reactions were then phenol-chloroform (1:1) extracted, extracted once with chloroform, and ethanol precipitated. Purified in vitro transcripts were analyzed by annealing 5′-^{32}P-labeled DNA primer (5′-AAAAGCAAGTGTAAG CAGCAGCTGCCCTGC-3′) at 65°C for 60 min (specific activity, 7,000 to 9,000 cpm/fmol) in a total volume of 30 μl. The volume was then increased to 150 μl by addition of the following to give the indicated final concentrations: 50 mM Tris hydrochloride (pH 7.5), 75 mM KCl, 10 mM dithiothreitol, 3 mM $MgCl_2$, 75 μg of actinomycin D per ml, and 0.5 mM each dATP, dGTP, dCTP, and dTTP. Two units of avian myeloblastosis virus reverse transcriptase (Promega Biotec) was added, and the mixture was incubated for 60 min at 37°C (38). Nucleic acids were ethanol precipitated, vacuum dried, dissolved in 5 M urea–50 mM NaOH–0.5 mM EDTA–0.025% bromophenol blue–0.025% xylene cyanole, and electrophoresed on a 10% polyacrylamide gel cast in 10 M urea and running buffer (90 mM Tris, 90 mM boric acid, 0.5 mM EDTA). The polyacrylamide gel was dried and visualized by autoradiography at −70°C, using Cronex film and a Cronex Lightning-Plus intensifying screen (E. I. du pont de Nemours & Co., Inc., Wilmington, Del.). Appropriately initiated RNAs produced a primer extension product 75 nucleotides long for OVEC vectors and a primer extension product 54 nucleotides long for the adenovirus major late template.

Renaturation of IBF. Renaturation of DNA-binding activity was performed basically as described by Hager and Burgess (21), with the inclusion of 0.1 mg of bovine serum albumin per ml in the gel slice elution buffer and the dilution buffer. Affinity-purified IBFαβ was precipitated with 10% trichloroacetic acid, and the pellet was washed with acetone and dissolved in SDS-polyacrylamide gel electrophoresis loading buffer. SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (30). The gel slice was crushed and shaken in 200 μl of elution buffer overnight at room temperature. The crushed acrylamide was then pelleted by centrifugation at 13,000 × *g*. The protein-containing supernatant was acetone precipitated with 10 volumes of acetone at −70°C for 30 min, and the air-dried pellet was dissolved in 6 M guanidine hydrochloride. The dissolved protein was then rapidly (1 s) diluted 50-fold with 10 mM HEPES (pH 8.0)–5% glycerol–50 mM NaCl–50 mM β-octylglucoside–7 mM β-mercaptoethanol–0.1 mg of bovine serum albumin per ml at 22°C and was allowed to renature for 2 to 24 h at 4°C before being assayed. Protease inhibitors at concentrations of 1 mM PMSF, 10 mM benzamidine, 5 μg of leupeptin per ml, and 0.7 μg of pepstatin per ml were included in all buffers for the experiments described in Fig. 5. For the mixing experiments, renatured IBFα was mixed with an excess of renatured IBFβ. The mixture was allowed to sit for 30 min before addition of the DNA and other components of the binding reaction. The buffer that the proteins were mixed in was the same as that described above, and the final guanidine hydrochloride concentration during the mixing was 60 mM.

Chymotrypsin digestions of IBF. Chymotrypsin (three times crystallized; Nutritional Biochemicals Corp., Cleveland, Ohio) digestion of IBF samples was performed in 10 mM HEPES (pH 8.0)–150 mM NaCl–5% (vol/vol) glycerol–0.1 mM EDTA–0.01% (vol/vol) Nonidet P-40–7 mM β-mercaptoethanol–10 mM $CaCl_2$–0.5 mg of bovine serum albumin per ml for 10 min at 23°C. The digestions were stopped by heating the sample very quickly to 100°C in an H_2O bath for 5 min. The digestion products were then cooled on ice, and residual IBF DNA-binding activity was analyzed by the mobility shift assay.

RESULTS

IES2 functions as a *cis*-acting sequence that stimulates transcription in vivo. We previously identified BHK cell nuclear proteins that interacted specifically with the RSV internal enhancer (29). These factors (IBF) showed specific interactions with the internal enhancer, as determined by the DNase I footprinting technique. These factors were also partially purified. From these studies, we determined that the factors bound to two sites within the RSV internal enhancer. The sites were between nucleotides 813 and 850 (IES1) and 856 and 878 (IES2) of the proviral genome, with the IBF-IES2 interaction being the stronger. The IBF-DNA interaction was initially identified, and IBF was partially purified by monitoring extracts and column fractions with a DNA retardation assay. With crude extracts, at least three protein-IES2 complexes were identified by using an oligonucleotide encompassing IES2 (OpvuII3′) as the probe for DNA mobility shift assays. These are referred to as complexes I, II, and III. Complex I has the greatest electrophoretic mobility, and complex III has the least.

To examine the significance of these protein-DNA interactions, we tested the ability of IES2 sequences to stimulate transcription as a *cis*-acting element in vivo. Duplications and multiplications of individual enhancer elements have previously been demonstrated to restore enhancer activity (17, 22, 23, 51). Thus, multimers of an oligonucleotide of the binding site IES2 were cloned into the *Sal*I site of the vector OVEC1 (49). OVEC1 contains a rabbit β-globin reporter gene and promoter (TATA box) but no known enhancer elements. Figure 1A shows the OVEC constructs containing one, two, three, or eight head-to-tail repeats of the IES2

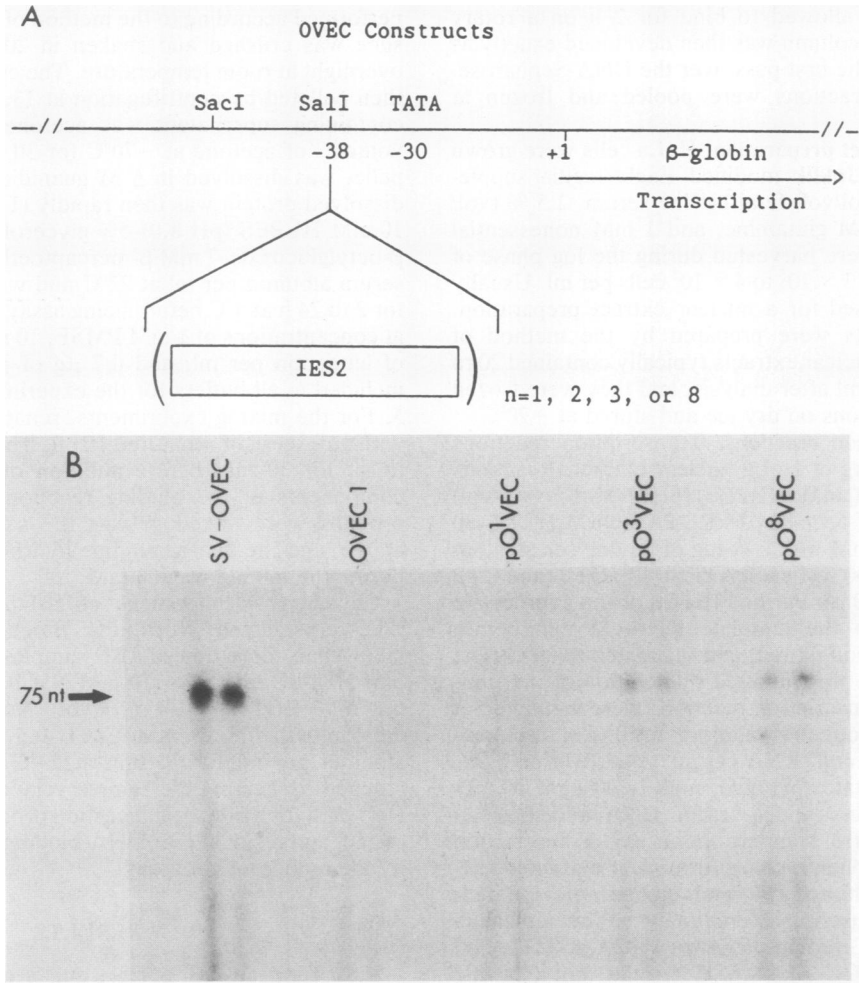

FIG. 1. IES2 functions as a *cis*-acting element in vivo. (A) Promoter region of OVEC1. Multiples of the IES2 inserts were cloned into the *Sal*I site. (B) The resulting plasmids (see Materials and Methods and Results) were transfected into BHK cells by the $CaPO_4$ procedure. At 48 h after transfection, cytoplasmic RNA was isolated, treated with RNase-free DNase I, and purified. Then 10 μg of RNA from each transfection experiment was annealed with an excess of ^{32}P-labeled primer, extended with reverse transcriptase, and electrophoresed on a 10 M urea-polyacrylamide sequencing gel as described in Materials and Methods. Each plasmid was transfected in duplicate, and the two independent RNA preparations were subjected to primer extension analyses. The results obtained in both experiments were then analyzed in the adjacent lanes of the gel. Extended products correspond to appropriately initiated RNA 5′ ends (arrow). nt, Nucleotides.

binding site. These were transfected into BHK cells, and the cytoplasmic RNA was isolated and analyzed by primer extension (Fig. 1B). For comparison, SV-OVEC, which contains the simian virus (SV40) enhancer inserted into OVEC1, was transfected in parallel. The results of one such transfection, done in duplicate, are shown in Fig. 1B. No extended product (i.e., no detectable transcription) was obtained by using RNA isolated from cells transfected with OVEC1 alone, whereas a very small amount of extended product, which could not be reproduced photographically, was obtained by using RNA samples prepared from cells transfected with the vectors containing one (pO^1VEC) or two (pO^2VEC; not shown) copies of the IES2 binding site. When a vector containing three copies of the IES2 binding site (pO^3VEC) was transfected into BHK cells and the RNA was analyzed, a much larger amount of extended product was visible. Transcription was enhanced to a level only a few fold lower than that seen for SV-OVEC. Finally, there was only a slight increase in transcriptional stimulation when the number of binding sites was increased from three to eight (pO^8VEC), which suggested that under these assay conditions enhancer function had been saturated. Each transfection was repeated at least three times, with the same results.

IES2 stimulates transcription in vitro. Since multiple copies of IES2 stimulated transcription in vivo, the OVEC1 constructs were tested for the ability to stimulate transcription in vitro. HeLa transcription extracts were chosen for these studies because they are well defined (14, 39–41). HeLa transcription extracts contain IBF-like proteins (unpublished observations), and we reasoned that such extracts would probably stimulate transcription in an IES2-dependent manner. Therefore, the OVEC vectors containing zero, one, three, or eight IES2 binding sites were tested for the ability to stimulate transcription in HeLa transcription extracts in vitro. Primer extension analyses of RNA synthesized in these transcription assays are shown in Fig. 2. Each template was incubated with increasing amounts of transcription extract. At low levels of extract (2 to 5 μl), a small amount of specific transcription from all templates (denoted by the 75-nucleotide marker) was detected, although little IES2-dependent stimulation of transcription was seen (Fig. 2). As the amount of extract was increased (5 to 10 μl), a

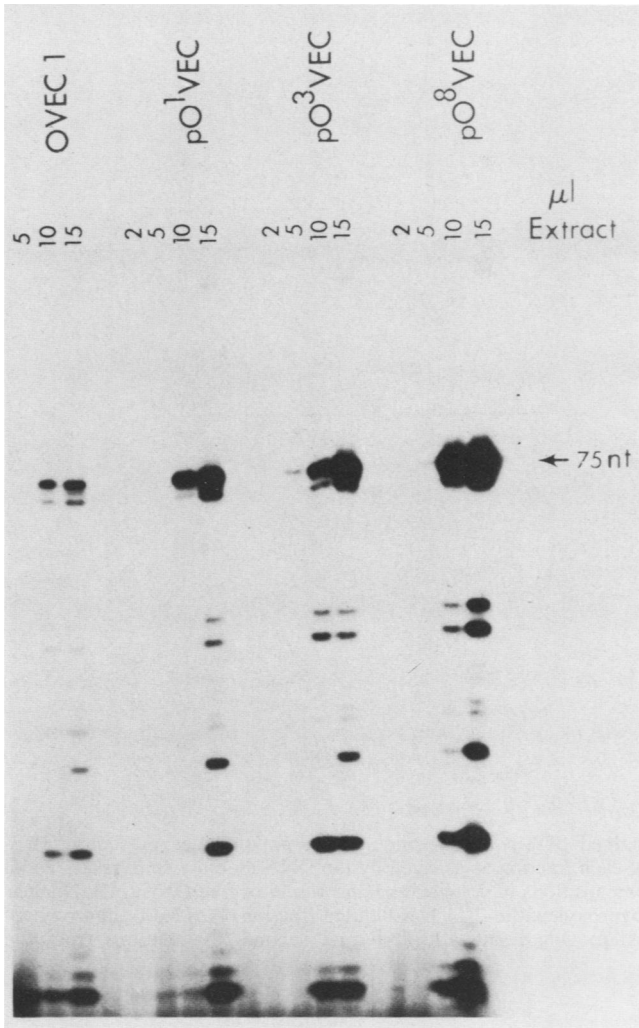

FIG. 2. Stimulation of transcription by IES2 functioning as a *cis*-acting element in vitro. The indicated plasmids were used as templates for an in vitro transcription experiment. Each template was incubated with 2, 5, 10, or 15 μl of HeLa nuclear extract as described in Materials and Methods. The RNA produced in these reactions was isolated and analyzed by primer extension. An extended primer 75 nucleotides (nt) in length (arrow) represents the correctly initiated transcript.

cis-acting sequence dependence for transcriptional stimulation was evident (compare OVEC1 with pO^{8}VEC). Transcription of the construct pO^{8}VEC resulted in the greatest amount of stimulation. As the number of IBF binding sites was decreased in the vector, transcription decreased, in agreement with results of the in vivo studies. Although the quantitative relationship between each OVEC construct in vivo did not exactly mimic that seen in vitro, the results indicated that the *cis*-acting sequence, IES2, specifically stimulated transcription in this in vitro system.

Purification of IBF. By using a combination of conventional and DNA affinity chromatography, the proteins that specifically bound to the RSV internal enhancer sequence IES2 were purified to near homogeneity (see Materials and Methods). Figure 3A shows a silver-stained SDS-polyacrylamide gel of the polypeptides present in the IBF-containing fractions; purified IBF designated OligoII. Figure 3B shows results of the mobility shift assays of protein fractions at each stage of purification. These mobility shift assays predominantly yielded two complexes, I and II.

A summary of the purification scheme is presented in Table 1. IBF was purified approximately 770,000-fold from the crude nuclear extract, with an overall yield of about 250 ng of IBF. The yield was estimated from the overall fold purification and agrees reasonably well with an estimate based on the intensities of the purified IBF compared with protein standard lanes on the silver-stained SDS-polyacrylamide gel (data not shown). The binding activity of the purified material gave the same qualitative shift as was seen in the crude extract (Fig. 3B).

After two passes over the DNA-affinity column, a major species consisting of a diffuse band with a molecular weight of approximately 40,000 and several minor peptides of molecular weights 68,000 and 30,000 were visible (Fig. 3A, lane OligoII). In addition, variable amounts of a peptide with a molecular weight of 20,000 were present in different purified preparations of IBF. The polypeptides with molecular weights of 20,000 (IBFα) and 40,000 (IBFβ) renatured to yield specific DNA-binding activities (see below). Purified IBF generated footprints identical to those previously reported (29) on the RSV internal enhancer (data not shown). A third pass over the DNA affinity resin resulted in no further purification and a very large loss of activity (results not shown). As shown below, IBFβ was most likely a proteolytic product of IBFα. Both proteins bound IES2 DNA specifically.

To determine which polypeptides present on the SDS-polyacrylamide gel of highly purified IBF were responsible for the IES2 DNA-binding activity, the following approach was used. IBF was concentrated by precipitation with 10% (wt/vol) trichloroacetic acid, divided equally between two equivalent lanes, and electrophoresed on an SDS-gel (Fig. 4B). The gel was cut between the two lanes, and one half was silver stained (Fig. 3A). The two halves were then aligned after the silver-stained gel swelled to the same dimensions as the unstained portion (by addition of water to the destain solution). Bands corresponding to the proteins visible by silver staining were excised individually from the unstained gel, and the rest of the gel lane was dissected into a series of slices (Fig. 4B). The diffuse band at 40 kDa was dissected into two gel slices. The proteins were eluted from the gel slices and renatured as described in Materials and Methods. The renatured proteins were then assayed for DNA-binding activity by the DNA retardation assay (Fig. 4A). The DNA retardation pattern (consisting of complexes I and II) that was obtained with the starting purified IBFαβ fraction (before purification by SDS-polyacrylamide gel electrophoresis) was used as a control. This assay demonstrated that renaturation of the band with an apparent molecular mass of 20 kDa generated a mobility shift similar to that found for complex I. The 40-kDa band yielded two complexes, one of which appeared to be equivalent to complex II. However, an additional, more slowly migrating complex (complex III) was also evident (Fig. 4A, lanes 7 through 9). The DNA-binding polypeptides of molecular weights 40,000 and 20,000 have been designated IBFα and IBFβ, respectively.

The appearance of a new complex (III) which closely resembled a complex that had been previously observed in variable but small amounts in crude nuclear extracts (29) was intriguing. Also, the fact that two complexes were present after renaturation of IBFα led us to hypothesize that complex II might be the result of proteolysis occurring during the renaturation of a single polypeptide species. In fact, the possibility that both complex II and complex I might be the

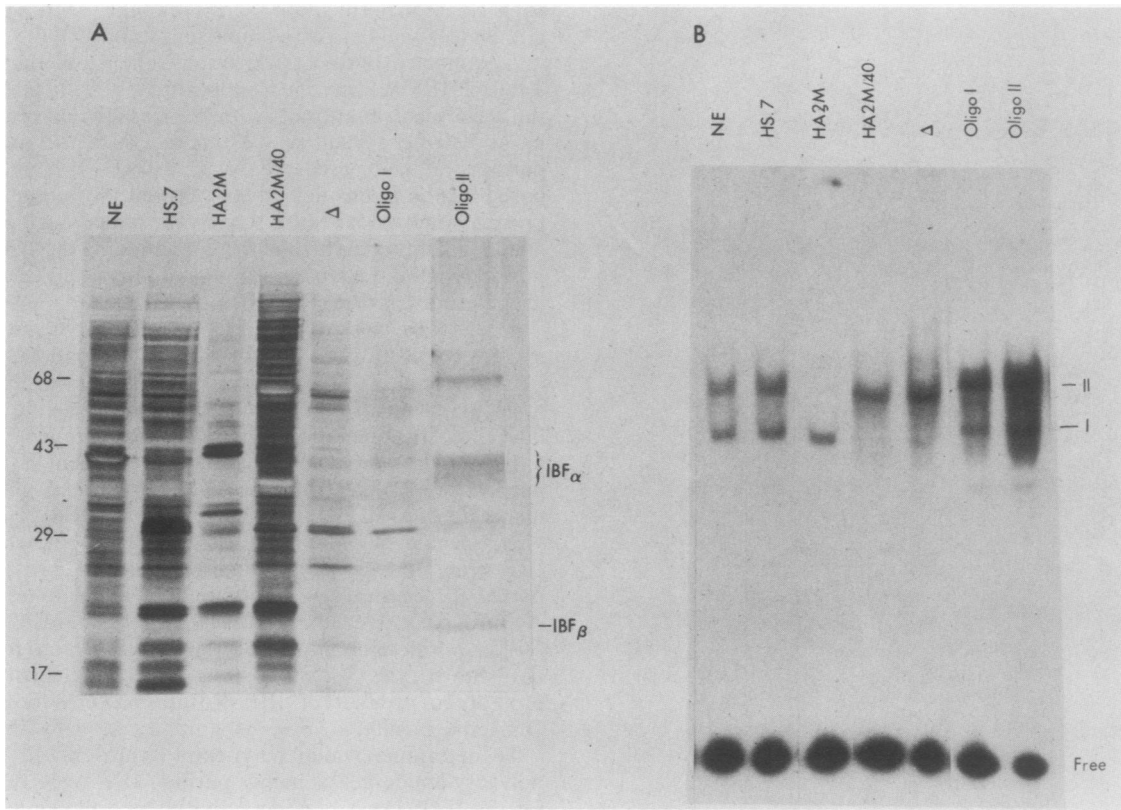

FIG. 3. Purification and DNA-binding activity of IBF. (A) Silver-stained SDS-polyacrylamide gel of polypeptides present in IBF-containing fractions at each stage of purification. (B) Binding activities of each fraction as assayed by the DNA mobility shift assay. Panel A shows mobilities of the protein standards (in kilodaltons); panel B shows positions of complexes I and II and of free DNA. NE, Nuclear extract; HS.7, heparin–Sepharose–0.7 M NaCl-eluted fraction; HA2M, hydroxylapatite–2 M NaCl-eluted fraction; HA2M/40, hydroxylapatite–2 M NaCl–40 mM sodium phosphate fraction; Δ, 100°C heat step; OligoI and -II, first- and second-pass elutions from the DNA-Sepharose affinity resin.

result of proteolysis seemed likely, since a one-way conversion of complex II into complex I was frequently seen during some purification procedures (data not shown). The appearance of complex II in an assay of proteins from gel slices 7 through 9 was revealed later to be the result of proteolysis (Fig. 5).

Also, small amounts of DNA-binding activity were renatured from the gel slices between IBFα and IBFβ, probably as the result of minor forms of proteolysis. These binding activities generated complexes that seemed to have slightly faster mobilities than did complexes II and III, which would also be consistent with the hypothesis that they are generated by proteolysis. Multiple minor forms of IBF have frequently been observed migrating between complexes II and I. Importantly, the other minor protein species observed on the SDS-polyacrylamide gel (the 68-kDa and the 30-kDa species in slices 3, 4, and 11) did not generate any DNA mobility shifts even in the absence of poly(dI-dC) · poly(dI-dC).

TABLE 1. Procedure for purifying IBFαβ

Purification step	Protein (μg)	Units	Sp act[a]	Yield (%)	Fold purification
Nuclear extract	200,000	ND[b]	ND	ND	1
Heparin-Sepharose	40,000	32,000	0.08	100	5
Hydroxylapatite[c]	5,000	30,500	6.1	95	8
100°C	1,700	30,500	17.9	95	3
DNA-Sepharose					
1	26[d]	15,000	5,800	47	640
2	0.21[d]	8,400	32,300	26	10

[a] Expressed as units of DNA binding activity of IBFαβ per microgram of protein; 1 U of activity equals 1 fmol of DNA bound.
[b] ND, Not determined.
[c] The 2 M NaCl–40 mM sodium phosphate fraction.
[d] Estimated by the fold purification.

IBF binds DNA as a dimer. In the experiment shown in Fig. 4, renaturation of the 40-kDa polypeptide (IBFα) yielded two complexes when assayed by the DNA mobility shift assay. Since we were highly suspicious that a proteolytic event was involved in this transition, a preparation containing IBFαβ was gel purified as before, with the inclusion of the protease inhibitors in the elution and renaturation buffers as described in Materials and Methods. The renatured materials from the 40-kDa band and the 20-kDa band obtained by this procedure were assayed by the DNA mobility shift assay. Lane 1 of Fig. 5 shows the mobility shift pattern of IBF before electrophoresis on the SDS-polyacrylamide gel used to purify IBFα and IBFβ for this experiment. Lanes 2 and 5 show the complexes obtained from renaturation of the 40-kDa (IBFα) and the 20-kDa (IBFβ) polypeptides, respectively. When renatured in the presence of protease inhibitors, only complex III was recovered from the 40-kDa polypeptide and complex I was formed exclusively

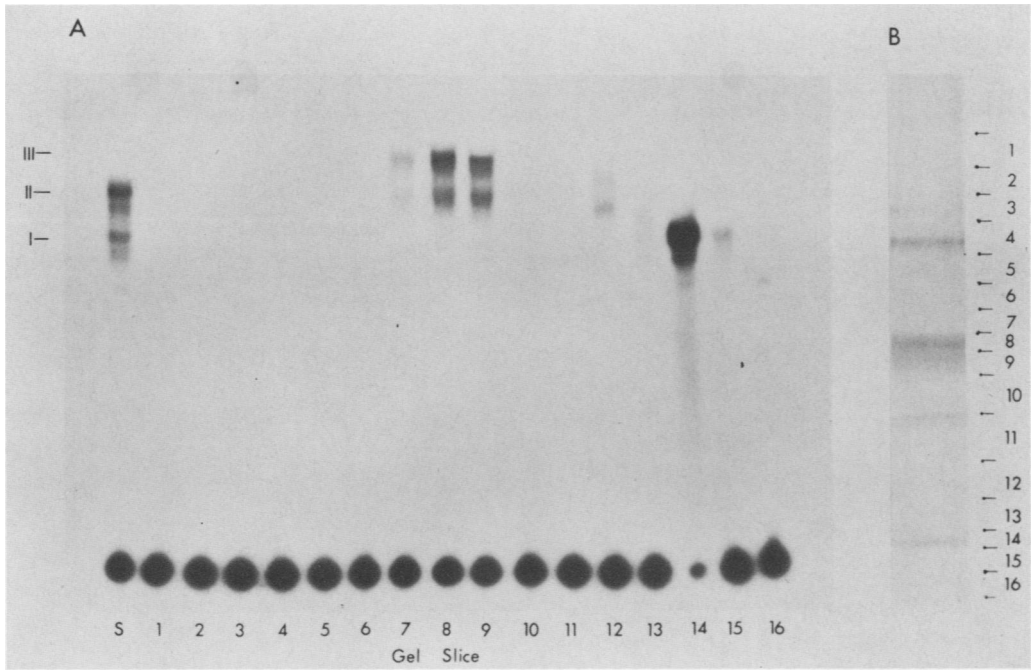

FIG. 4. Identification of protein species corresponding to IBFα and IBFβ. (A) Two parallel lanes containing IBFαβ purified by two passes over the DNA affinity column were electrophoresed on an SDS-polyacrylamide gel. The DNA mobility shift pattern of the starting material is shown in lane S. The gel was cut between the lanes, and one half was silver stained (B). After the silver stained gel swelled to the size of the unstained gel, slices were dissected from the unstained gel and numbered as shown on the right. Each slice was then crushed, and the proteins therein were eluted by diffusion overnight as described in Materials and Methods. The renatured proteins were assayed by the DNA mobility shift assay. No poly(dI-dC) · poly(dI-dC) was used in the assay. Positions of complexes I, II, and III are indicated on the left.

from the 20-kDa polypeptide. Complex II was not recovered from any of the other gel slices. However, when IBFα was mixed with an excess of IBFβ at low ionic strength (lane 8), complex II was re-formed and the DNA mobility shift pattern was the same as that of the starting material (lane 1). The relative amounts of IBFα and IBFβ that were recovered for this experiment were not determined, although more IBFβ than IBFα was present during the mixing. This could account for the lack of complex III formation. Another possible explanation is that there is a preference for the dimerization of the proteolyzed forms.

All of the observed complexes were demonstrated to bind DNA specifically. Lanes 2, 5, and 8 of Fig. 5 show binding in the absence of competitor; lanes 3, 6, and 9 show efficient competition with a 100-fold molar excess (20 ng) of IES2; and lanes 4, 7, and 10 show a lack of competition with a 100-fold molar excess (20 ng) of a NFI binding site (26). The simplest model consistent with these data is that complex II is formed by DNA interacting with a dimer (IBFαβ) composed of one subunit each of IBFα and IBFβ. It is possible that higher multimers could be involved; however, this would be expected to yield a range of additional complexes, and none have been detected. This model also predicts that complex I contains IBFββ and that complex III contains IBFαα.

IBFβ is formed by proteolysis of IBFα. To formally test the hypothesis that the multiple forms of retarded DNA complexes were indeed the result of proteolytic processes, SDS-polyacrylamide gel-purified, renatured IBFα was subjected to digestion with small amounts of trypsin or chymotrypsin. No stable trypsin-resistant fragments containing an intact DNA-binding domain were found (unpublished results). However, digestions of IBFα with increasing amounts of chymotrypsin (0 to 20 ng/ml) led to changes in complex migration (Fig. 6). A preparation that started out greatly enriched in IBFαα (complex III) was sequentially degraded into IBFαβ (complex II) and then into IBFββ (complex I). Figure 6 also shows results of an assay of the starting material (containing IBFα and IBFβ) before application to the SDS-polyacrylamide gel for purification of IBFα. Hydroxylapatite-purified IBFβ is shown as a standard for complex I.

Purified IBF specifically stimulates RNA polymerase II-mediated transcription in vitro. The experiment represented in Fig. 7 demonstrates the effect on transcription from various templates when HeLa transcription extracts were supplemented with purified IBFαβ. Since significant amounts of endogenous IBF were present in these HeLa nuclear extracts (see above), the amount of extract used for transcription was minimized to reduce endogenous HeLa IBF-mediated stimulation of transcription (Fig. 2). At these reduced levels of extract, endogenous IBF was limiting. Therefore, addition of exogenous purified IBF should have enhanced transcription, and the enhancement should have depended on the presence of multiple *cis*-acting IES2 binding sites (Fig. 2). Each set of lanes in Fig. 7 shows the results of primer extension analyses of the RNA transcribed from the indicated templates, and the sizes of each extended primer are given. Purified IBFαβ was added to each template DNA and allowed to bind for 15 min at 30°C; transcription extract was then added, and the incubation was continued at 30°C for 1 h. Transcription reactions were performed for each template in the presence or absence of added IBFαβ. The reaction was stopped as described in Materials and Methods. Also included at the termination of each reaction was a tracer amount of ^{32}P-labeled DNA (250 base pairs, internal standard) to account for any losses of nucleic acids during sample manipulation.

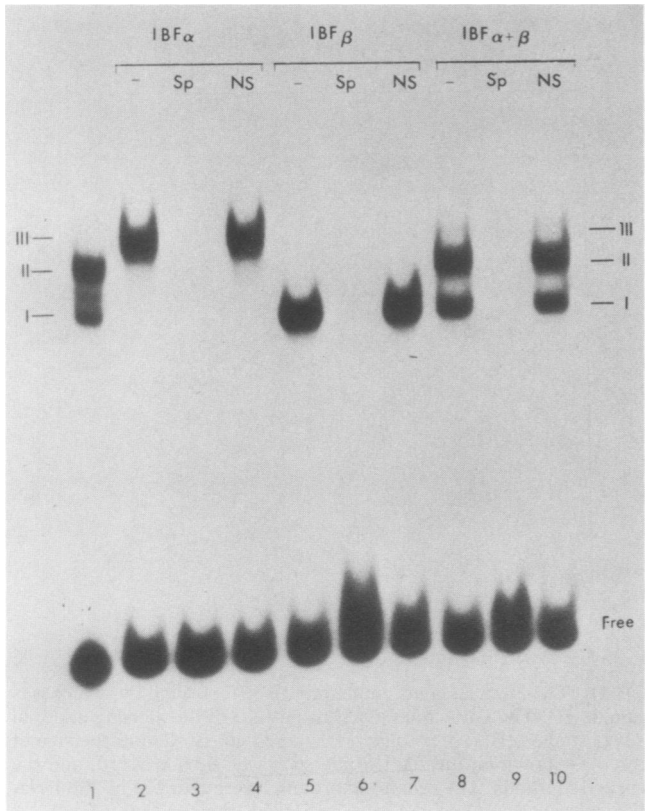

FIG. 5. Complex II is composed of multiple distinct components. IBFα and IBFβ were gel purified. Lane 1 shows the DNA retardation pattern obtained from the starting material used to gel purify IBFα and IBFβ. To demonstrate the specificity of each protein-DNA interaction, competitions with a 100-fold molar excess of unlabeled oligonucleotide are shown: −, no competitor; NS, nonspecific competitor (NFI binding site); SP, specific competitor (OpvuII3′). Assays were performed with 0.2 ng of ^{32}P-labeled OpvuII3′ and 5 ng of poly(dI-dC) · poly(dI-dC) in a final volume of 20 μl. Positions of complexes I, II, and III and of free DNA are indicated.

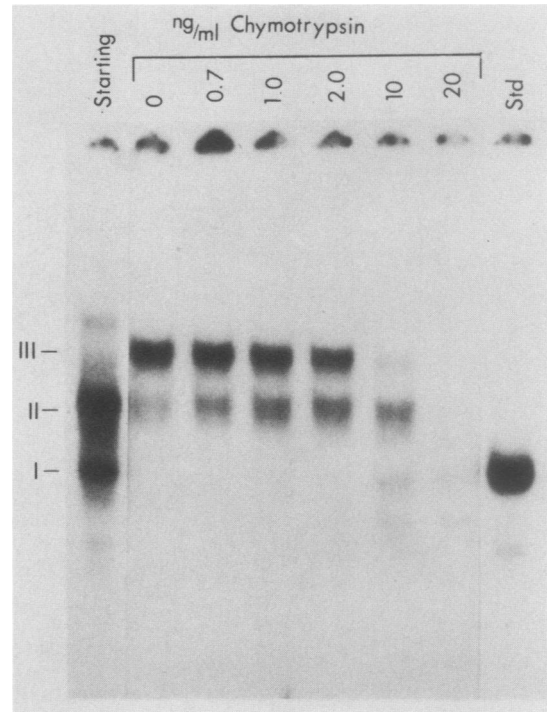

FIG. 6. Demonstration that chymotrypsin proteolysis of IBFα generates polypeptides which give DNA mobility shifts similar to those seen in crude nuclear extracts. SDS-polyacrylamide gel-purified IBFα was treated with various amounts of chymotrypsin, and the digested protein was assayed for DNA-binding activity by the DNA retardation assay. The retardation assay of purified IBFαβ used as a starting material for the preparation of IBFα is shown (Starting). As a reference for complex I, hydroxylapatite-purified IBFβ is shown (Std). Positions of complexes I, II, and III are shown on the left.

IBFαβ did not stimulate transcription from templates containing either adenovirus major late promoter or the SV40 enhancer. Addition of purified IBF to a reaction in which OVEC1 was the template also showed no stimulatory effect on the level of transcription. In fact, a small decrease in transcription was evident when the factor was added, although this could be accounted for by a corresponding decrease in the amount of recovered internal standard. Addition of purified IBFαβ to the reaction in which pO^{3}VEC was the template showed a significant increase in transcription. This increase, in the absence of a stimulatory effect of IBF for the other templates, indicated that the factor identified as IBF and purified by the gel mobility shift assays was indeed a specific *trans*-acting transcription factor.

DISCUSSION

The DNA-binding protein (IBF) that interacts with at least one DNA element (IES2) within the RSV internal enhancer has been purified and demonstrated to stimulate transcription in an in vitro system. In addition, IES2 has been shown to stimulate transcription as a *cis*-acting sequence both in vivo and in vitro when multiple copies are present in a vector containing the rabbit β-globin promoter and β-globin reporter gene (49). Previous workers have demonstrated that individual enhancer elements alone do not stimulate transcription in vivo when only a single copy is present, although reiteration of these elements within the vector reestablishes the enhancer function of these elements. With this approach, it has been shown that when three (or eight) head-to-tail repeats of the RSV IES2 sequence are placed upstream of the β-globin gene of the vector OVEC1, enhancement of transcription only a few fold less than that seen for the SV40 promoter in the same vector is seen. When the same three head-to-tail inserts are present in front of the chloramphenicol acetyltransferase gene of pSV1-CAT, approximately the same degree of enhancement is observed as with the entire 519-base-pair internal enhancer (data not shown).

When IES2-containing OVEC vector constructs were used in in vitro transcription assays, the same qualitative pattern of transcriptional enhancement was seen at the higher levels of transcription extract used. IES2-dependent stimulation of transcription was not evident at the lower levels of transcription extract, although a basal level of transcription was still evident. This result indicates that at low levels of transcription extract, HeLa IBF must be the limiting protein required for IES2-dependent transcriptional stimulation. The addition of purified IBFαβ to transcription reactions performed under these IBF-limiting conditions demonstrated that IBFαβ was able to stimulate transcription only with templates that contained repeated binding sites for IBF. The specificity of this effect was demonstrated by the

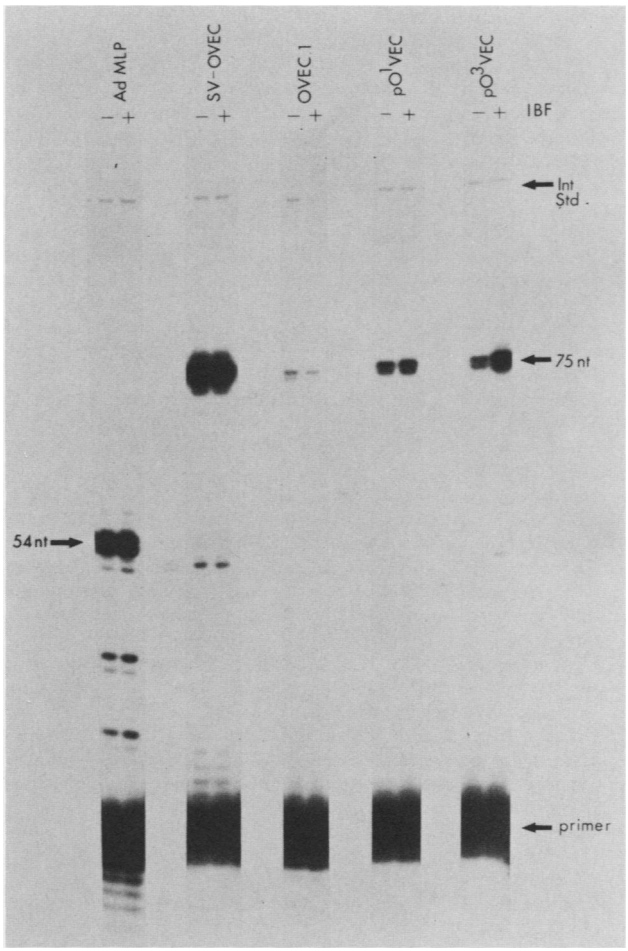

FIG. 7. Demonstration that highly purified IBFαβ specifically enhances transcription. The indicated templates were used for in vitro transcription reactions in the presence (+) or absence (−) of IBFαβ that had been purified by two passes over the DNA affinity column. Sizes of the excess primer and the extended primers (in nucleotides [nt]) are indicated. The extended primers were analyzed as described in Materials and Methods. At the termination of each transcription reaction, a tracer quantity of ^{32}P-labeled DNA was added as an internal standard (Int Std) to monitor recoveries of nucleic acid through the purification and analysis procedures. The autoradiograph was trimmed to remove blank film between the internal standard and the 75-nucleotide extended product. AdMLP, Adenovirus major late promoter.

fact that no IBF-dependent transcriptional enhancement was seen for either the adenovirus major late promoter or the SV40 enhancer-containing templates.

If a DNA affinity-purified preparation of IBF that generated primarily (95%) complex II in the gel retardation assay was electrophoresed on an SDS-polyacrylamide gel, several well-resolved bands were identified by silver staining of the gel. When these proteins were eluted from the gel, renatured, and analyzed by the DNA mobility shift assay, two major polypeptides that specifically bound IES2 DNA were identified. The 20-kDa (IBFβ) band was found to yield complex I, and the major, 40-kDa (IBFα) band was found to yield the more slowly migrating complex III. Relatively little complex II was obtained from either band. Mixing experiments demonstrated that when the renatured 20-kDa protein (IBFβ) was mixed with the renatured 40-kDa protein band (IBFα), the original DNA-binding behavior was obtained (formation of complexes I and II). Such a result indicates that complex II is most likely composed of two different polypeptides (IBFα and IBFβ) interacting with IES2.

Since preparations of protein that initially gave rise primarily to complex II were gradually converted into a form that gave rise to complex I, we reasoned that proteolysis might have generated these multiple forms. To address this question, limited protease digestions were performed on gel-purified and renatured IBFα. The protease digestion data demonstrated that digestion of IBFα with increasing amounts of chymotrypsin produced proteolytic fragments which, when assayed by the DNA mobility shift assay, resulted in the appearance of the more rapidly migrating complexes. These complexes comigrated with complexes II and I; in addition, a new, even more rapidly migrating complex was generated. Native IBF is most likely a homodimer consisting of two identical α subunits, although the proteolyzed form may be present in vivo as well. The various complexes obtained in gel mobility shift assays are therefore a consequence of DNA binding to the various combinations of intact and proteolyzed monomers.

The binding of IBF to DNA as an apparent homodimer is not surprising, considering that its binding site consists of an inverted repeat of 15 of 16 nucleotides. Many procaryotic DNA-binding sites bind to DNA as dimers (reviewed in reference 35). The yeast GCN4 protein has also been demonstrated, by a method similar to the one used here, to bind to its recognition sequence as a homodimer (24). Also, other members of the CCAATT binding family have been demonstrated to bind DNA as heterodimers; in these cases, neither subunit alone specifically binds to DNA (7). Finally, Chodosh et al. have demonstrated that yeast HAP2 and HAP3 proteins are both required for binding to their DNA recognition sequence and are functionally interchangeable with the human CCAAT-binding protein subunits (9).

One final point that remains to be addressed is the relationship of IBF to other transcription-enhancer factors. Similarities between IBF and the rat liver DNA-binding protein EBP20 (25) are intriguing. Both factors are heat stable, and there are obvious sequence similarities between a CCAAT binding site and the IBF binding site. The EBP20-CCAAT binding site in the herpesvirus thymidine kinase gene (20) is given in the top line, the IBF binding site is given on the lower line, and sequence identities are indicated by asterisks:

```
5′-TTCGCCAATGACAAG-3′
   *   ****   *
5′-GGCTGCAATTGCGCC-3′
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It has previously been shown that the SV40 enhancer (nucleotides 5235 to 270), to which EBP20 binds, does not effectively compete for binding of IBF to IES2 (29). Moreover, since purified IBF does not enhance in vitro transcription from SV-OVEC, we believe that IBF is a different, although perhaps related, member of this family of proteins. Carlberg et al. (6) have recently demonstrated that EBP20 also can bind to the Fujinami sarcoma virus internal enhancer. Since this internal enhancer has never been tested for activity in a liver cell line, EBP20 may not be the protein responsible for transcriptional activation in the fibroblastlike BHK cell line. It is highly likely that IBF and EBP20 are related proteins and perhaps members of the same family of DNA-binding proteins. We are currently engaged in experiments to determine the relationship between the hamster fibroblast IBF and the rat liver EBP20.

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