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cDNA Cloning of Transcription Factor E4TF1 Subunits with Ets and Notch Motifs

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E4TF1 was originally identified as one of the transcription factors responsible for adenovirus E4 gene transcription. It is composed of two subunits, a DNA binding protein with a molecular mass of 60 kDa and a 53-kDa transcription-activating protein. Heterodimerization of these two subunits is essential for the protein to function as a transcription factor. In this study, we identified a new E4TF1 subunit, designated E4TF1-47, which has no DNA binding activity but can associate with E4TF1-60. We then cloned the cDNAs for each of the E4TF1 subunits. E4TF1 was purified, and the partial amino acid sequence of each subunit was determined. The predicted amino acid sequences of each cDNA clone revealed that E4TF1-60 had an ETS domain, which is a DNA binding domain common to *ets*-related transcription factors. E4TF1-53 had four tandemly repeated notch-ankyrin motifs. The putative cDNA of E4TF1-47 coded almost the same amino acid sequences as E4TF1-53. Three hundred and thirty-two amino acids of the N termini of E4TF1-47 and -53 were identical except for one amino acid insertion in E4TF1-53, and they differ from each other at the C terminus. These three recombinant cDNA clones were expressed in *Escherichia coli*, and the proteins behaved in the same manner as purified proteins in a gel retardation assay. Nucleotide and predicted amino acid sequences were highly homologous to GABP-α and -β, which is further supported by the observation that GABP-specific antibody can recognize human E4TF1.

Regulation of transcription is an important step in gene expression, and it is controlled by various proteins including sequence-specific DNA binding proteins, general transcription factors, and RNA polymerase II. To understand the molecular mechanisms of the regulation, it is necessary to elucidate protein-protein interactions of these transcription factors.

Regulation of adenovirus genes provides a good model with which to understand transcription regulation, because cellular transcription factors and viral factors interact. Adenovirus E1A is a multipotent protein as well as a transcription regulator (2, 5). During adenovirus infection, E1A stimulates adenovirus early gene transcription, but it does not act directly, since it does not recognize a specific DNA sequence. Thus, adenovirus E1A-dependent activation of transcription provides a useful model with which to understand the protein-protein interactions of transcription factors.

The adenovirus E4 gene, located at the right end of the genome, is an early gene that is transactivated by E1A. Deletion analysis of the promoter has revealed the region responsible for E1A transactivation (6, 19, 20). The region from −140 to −173 bp upstream of the transcription initiation site is responsible for the activation, and we identified two transcription factors, E4TF1 and E4TF3/ATF, which recognize the specific sequences in this region.

E4TF3/ATF recognizes four sites in the E4 promoter (29). This motif can be found in other promoters of adenovirus early genes such as E1A, E2, and E3 (9), and E4TF3/ATF is supposed to be a mediator of transactivation by the E1A protein (16). E4TF3/ATF is composed of several type of transcription factors. Some of them can form hetero- and homodimers and function as a transcription activator (28).

E4TF1 recognizes the sequence 5′-CGGAAG-3′ in the E4 promoter. This site is located adjacent to the E4TF3/ATF binding site. E4TF1 is composed of two distinct subunits of 60 and 53 kDa. In vitro transcription and gel retardation have revealed that the 60-kDa subunit (E4TF1-60) has DNA binding activity but not transcription-stimulating activity in vitro. The 53-kDa subunit (E4TF1-53) does not have DNA binding activity. However, it can interact with E4TF1-60, and this interaction stimulates transcription in vitro (29).

In order to understand the structure and function of E4TF1, we tried to isolate cDNA clones of each subunit. In this study, we cloned the cDNAs of E4TF1 subunits and expressed them in *Escherichia coli*. We confirmed by gel retardation assays that their DNA binding properties were the same as those of affinity-purified proteins from HeLa cell extracts. In addition, we identified another protein which interacted with E4TF1-60.

MATERIALS AND METHODS

Purification and amino acid sequence determination of E4TF1 subunits. E4TF1 was purified from HeLa cell nuclear extracts as described previously or by using DNA affinity latex particles (8, 11). About 20 μg of affinity-purified E4TF1 was precipitated with 20% trichloroacetic acid, and each subunit was fractionated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The gel was stained with Coomassie brilliant blue, and then the stained band corresponding to E4TF1-60 was excised from the gel and incubated in digestion buffer (100 mM Tris-HCl [pH 9.0], 0.1% SDS) in the presence of 2 μg of lysyl-endopeptidase per ml. After 16 h at 37°C, the supernatant was recovered and

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60kd	#1	MQLHGIAQPVTAV
	#2	LNQPELVAQK
	#3	NILEIV
53kd	#4	MTALH(W)ATEHNHQEVVELL(I)
	#5	YGADVHTQSK
	#6	TAFDISIDNGNEDLAEILQI
	#7	-Q-IEIIENRVE-A
	#8	QLDEANREAQ
	#9	(K)EQEAE

FIG. 1. Amino acid sequences from digested peptides of E4TF1-60 and -53.

injected onto a C18 column (4 by 30 mm; Brawnlee Labs). To prevent SDS from contaminating the reverse-phase column, DEAE-Sepharose Fast Flow resin was used as a precolumn as described previously (12). The column was equilibrated with the weak mobile phase, 0.1% Trifluoroacetate, and eluted with a 70% acetonitrile linear gradient of the eluting mobile phase (0.1% trifluoroacetate and 70% acetonitrile). The gel slice containing the E4TF1-53 band was digested in the presence of urea, and peptides were separated on a C18 column as described above except that a DEAE precolumn was not used. Some of the peak peptides were sequenced on an Applied Biosystems 477-A protein sequencer with the standard cycle described by the manufacturer.

cDNA cloning of E4TF1. A HeLa cDNA library was constructed in lambda gt10 by using oligo(dT) or random hexanucleotide primers according to the basic protocol (21), and the library was propagated in *E. coli* C600hfl. Degenerated oligonucleotides deduced from the amino acid sequences were synthesized and used as hybridization probes with which to isolate cDNA clones from the HeLa cDNA library. To screen E4TF1-60 cDNA, oligonucleotides capable of encoding peptide sequence 1 (Fig. 1) were synthesized by using a neutral base, inosine, at the degenerated base by an Applied Biosystems 392 DNA synthesizer. The sequence 5′-ACIGCIGTIACIGGITGIGCIATICCATGIAGITGC AT-3′ was labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. This labeled probe was hybridized in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt's solution, 0.1% SDS, and 0.1 mg of single-stranded DNA per ml at 45°C. Hybridization was performed for 16 h. Membranes were washed twice for 30 min with 2× SSC–0.1% SDS and then twice for 15 min with 0.1× SSC–0.1% SDS. The same procedure was used to screen E4TF1-53. Oligonucleotides encoding peptide sequence 4 (Fig. 1) were used as probes for screening the HeLa cDNA library. The sequence of the probe was 5′-ATGACIGCICTICA(TC) TGGGCIACICA(AG)CA(TC)AA(TC)CA(TC)CA(AG)GA (AG)GTIGTIGA-3′.

DNA sequencing. Positive cDNA clones were recloned into pUC119 by using endogenous restriction sites. Sequences were determined by dideoxy chain termination (22) with Sequenase (U.S. Biochemicals).

Gel retardation assay. Binding reaction and gel electrophoresis were performed as described by Watanabe et al. (30), except that electrophoresis was run at 4°C. The DNA probes containing the E4TF1 binding site were prepared from plasmid pUCE4-20 as described previously (29). For the competition assay, DNA fragments containing the E4TF3 and NF1 binding sites were prepared as described previously (8, 28) and used as nonspecific competitors. For specific competitors, DNA fragments similar to the labeled DNA probe were used.

Immunoblotting. The affinity-purified E4TF1 fraction was fractionated on an SDS–8% polyacrylamide gel. After the electrophoresis, the gel was electrotransferred (50-mA constant current) onto a nylon membrane (Biodyne) for 1.5 h at 4°C. The membrane was incubated for 1 h at room temperature in blocking buffer (50 mM Tris-HCl [pH 8.0], 5% nonfat dry milk). The membrane was then incubated at room temperature overnight in blocking buffer containing antiserum to GABP-α or -β. Antisera to GABP-α and -β were provided by S. L. McKnight's group (Carnegie Institution of Washington). After incubation, the membrane was washed three times with a 500-ml solution of TBS-T (20 mM Tris-HCl [pH 7.6], 137 mM NaCl).

Recombinant E4TF1 expression in *E. coli*. The cDNA clone of E4TF1-60 was recloned into an expression vector as follows. Polymerase chain reaction or synthesized DNA was used to obtain 5′ and 3′ ends of cDNA clones in which *Nco*I and *Bam*HI sites were introduced. These DNA fragments had a *Nco*I or *Bam*HI site in one site and endogenous restriction sites on the others. Most of the open reading frame of the cDNA of E4TF1-60 was excised from a cDNA clone in pUC119 by using endogenous restriction sites. These three DNA fragments were ligated into the *E. coli* expression vector pET3d (25), which was cleaved with *Nco*I and *Bam*HI. To reclone E4TF1-53 and -47, DNA fragments from the 5′ end of the open reading frame to the *Hin*dIII site were synthesized by using a DNA synthesizer. The 3′ end of the DNA fragment was obtained by polymerase chain reaction between the *Eco*RI site and the 3′ end. The internal region between the *Hin*dIII and *Eco*RI sites of the open reading frame was excised from the cDNA of E4TF1-53 and -47.

These expression plasmids were transformed into *E. coli* BL21 (DE3). Once exponentially growing bacteria at 37°C reached an optical density at 600 nm of 0.6, they were induced with 2 mM isopropyl thiogalactoside and incubated at 37°C for 4 h. The bacteria were harvested by centrifugation, washed with phosphate-buffered saline, suspended in buffer A (10 mM Tris-HCl [pH 7.9], 100 mM KCl, 0.5 mM EDTA, 1 mM $MgCl_2$, 2 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 2 mM $CaCl_2$, 1 μg of lysozyme per ml), and incubated for 40 min at 4°C with gentle agitation. The solution was then frozen and thawed five times, and the DNA was fragmented with a syringe attached to a 25-gauge needle. The extracts were directly loaded for SDS-PAGE, and the desired proteins were excised from the gel and renatured as described previously (30). Usually, *E. coli* cultured in 5 ml of Luria broth LB was suspended in 200 μl of buffer A, and 10 μl of extract was loaded for the SDS-PAGE. The renatured protein was suspended in 100 μl of 0.05TGKEDN (50 mM Tris-HCl, 20% glycerol, 0.05 M KCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1% Nonidet P-40).

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper have been submitted to the DDBJ, EMBL, and GenBank nucleotide sequence data bases with the following accession numbers: D13316 (human E4TF1-47 gene), D13317 (human E4TF1-53 gene), and D13318 (human E4TF1-60 gene).

RESULTS

Purification of E4TF1-47 by using DNA affinity latex particles. Large amounts of E4TF1 were obtained by DNA affinity latex particle purification (8). The particles enabled

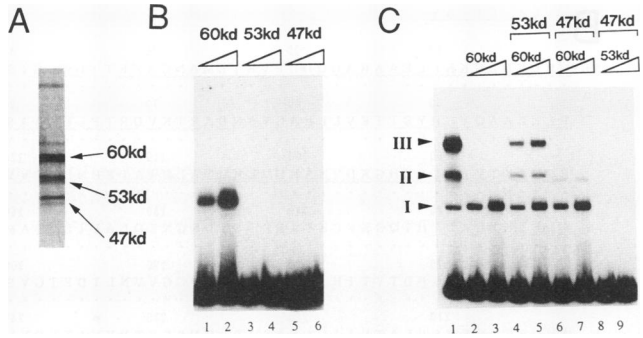

FIG. 2. Affinity-purified E4TF1 and the complex formation ability of each subunit. (A) E4TF1 was purified directly from HeLa nuclear extracts by using DNA affinity latex particles. The eluate was fractionated by SDS-PAGE and stained with silver. Each subunit (60, 53, and 47 kDa) is indicated. (B) DNA binding activity of the each renatured proteins. About 1.0 μg of affinity-purified fraction was separated by SDS-PAGE and then the 60-, 53-, and 47-kDa polypeptides were excised and renatured as described previously. Each polypeptide was suspended in 100 μl of renaturing buffer and dialyzed. Lanes 1, 3, and 5, 0.5 μl of renatured protein; lanes 2, 4, and 6, 1.5 μl of renatured protein in the gel retardation assay. (C) The E4TF1-60–DNA complex (complex I) was further shifted by adding E4TF1-53 or the 47-kDa protein. Lane 1, 0.5 μl of affinity-purified E4TF1; lanes 2, 4, and 6, 0.5 μl of renatured E4TF1-60; lanes 3, 5, and 7, 1.5 μl of renatured E4TF1-60; lanes 4 and 5, 1.0 μl of E4TF1-53; lanes 6 to 9, 1.0 μl of 47-kDa protein; lane 8, 0.5 μl of E4TF1-53; lane 9, 1.5 μl of E4TF1-53.

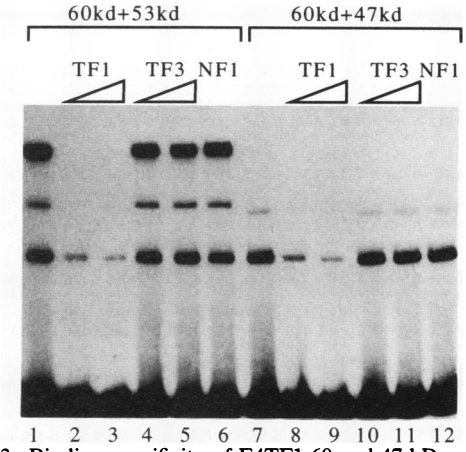

FIG. 3. Binding specificity of E4TF1-60 and 47-kDa complexes. Renatured 60- and 47-kDa proteins were mixed and tested for their DNA binding specificities. A total of 1.5 μl of 60-kDa protein and 1.5 μl of 47-kDa protein was used (lanes 7 to 12) in the gel retardation assay. Competitor DNAs were added where indicated. Triangles indicate increasing concentrations of competitors, 5 and 10 M excess. A 10 M excess of competitor DNA with a NF-1 binding site was added (NF1). As a control, the same competitors were used in a gel retardation assay that contained 1.5 μl (each) of E4TF1-60 and E4TF1-53 (lanes 1 to 6).

direct purification of E4TF1 from crude cell extracts. Figure 2A shows that three major E4TF1 proteins were copurified from the crude nuclear extract. Those with molecular masses of 60 and 53 kDa were E4TF1-60 and E4TF1-53, respectively, as identified previously (30). The third protein, with a molecular mass of 47 kDa, was copurified. The 47-kDa protein in addition to E4TF1-60 and E4TF1-53 was excised from the SDS gel, renatured, and examined for its DNA binding activity by using gel retardation assays. As shown in Fig. 2B, it could not bind DNA by itself. However, the 47-kDa protein interacted with E4TF1-60, as did E4TF1-53 (Fig. 2C). E4TF1-53 interacted with E4TF1-60 to produce two more complexes (complex II and III) in addition to that formed with DNA and E4TF1-60 alone (complex I). On the other hand, the 47-kDa protein could form only one more complex. The mobility of this complex was slightly different from that of complex II. This difference might reflect their molecular masses. The binding specificities of these complexes were examined by competition studies. All these complexes were inhibited only when the specific competitor was added (Fig. 3). E4TF3 and the NF1 binding site had no effect on the formation of these complexes. Thus, it could be concluded that this 47-kDa protein was similar to E4TF1-53. Therefore, it was designated E4TF1-47.

Isolation of cDNA clones of E4TF1. In order to study the structure and function of E4TF1, we tried to isolate a cDNA clone for each E4TF1 subunit. E4TF1 was purified directly from HeLa cell crude nuclear extracts as described previously. About 20 μg (each) of E4TF1-60 and -53 was purified from 600 mg of HeLa nuclear extracts. Each subunit was fractionated by SDS-PAGE and digested with lysyl-endopeptidase. These products were separated by high-performance liquid chromatography (HPLC), and some of the recovered oligopeptides were sequenced by an automated amino acid sequencer. Nine amino acid sequences were obtained from E4TF1-60 and -53, as shown in Fig. 1. Because of the limited amount of E4TF1-47, we could not obtain an amino acid sequence.

Degenerated oligonucleotides deduced from the determined amino acid sequences were synthesized and used as hybridization probes to isolate cDNA clones of E4TF1-60 from a HeLa cell cDNA library. Oligonucleotides that encoded peptide sequence 1 (Fig. 1) were used to screen 3×10^5 independent clones, and three E4TF1 clones were plaque purified. These cDNA clones were recloned into vector pUC119 and sequenced according to standard methods. Because none of the clones encoded the N terminus of the open reading frame, a cDNA library prepared from HeLa cell mRNAs cloned into lambda gt10 by using random primers was screened. Three independent clones containing the putative N terminus of the protein were obtained. Each clone was recloned into vector pUC119 and sequenced. The clones had an open reading frame of 1,362 bp predicting a protein of 454 amino acids. The amino acid sequences of all three peptides were found in the deduced sequence. The molecular mass of this clone was predicted to be 51 kDa, though the molecular mass of affinity-purified E4TF1-60 was estimated to be 60 kDa on the basis of SDS-PAGE fractionation.

Oligonucleotides that encoded peptide sequence 4 (Fig. 1) were used to isolate cDNA clones of E4TF1-53 from a HeLa cell cDNA library. Five positive clones were obtained, and they were divided into two classes based on partially different sequences in their open reading frames. One class of the open reading frame encoded a protein of 383 amino acids. Four other amino acid sequences determined from digested peptides 5 to 9 were also found in this open reading frame (Fig. 4). Another class of cDNA clone had a shorter open reading frame. This clone encoded a protein of 347 amino acids with the same 332-amino-acid sequence as that of the 383-amino-acid protein at the N terminus but differs in a 15-amino-acid sequence at the C terminus (Fig. 4). This open reading frame was shorter than E4TF1-53 by 36 amino acids

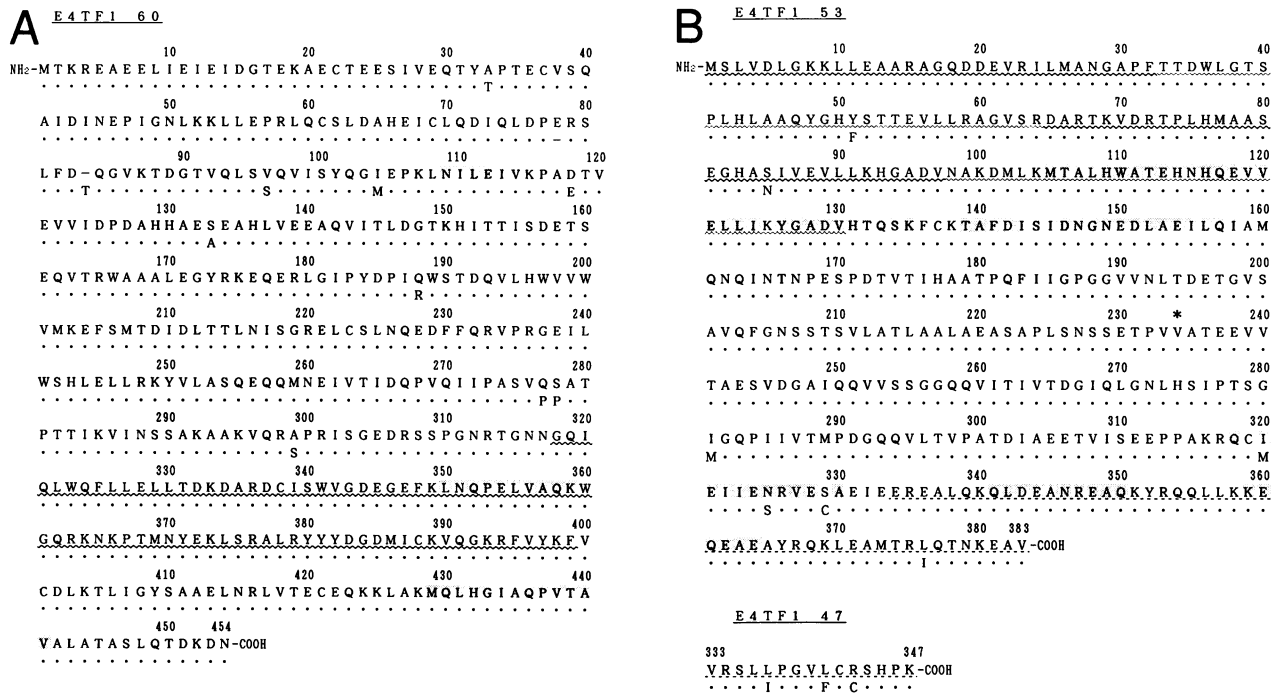

FIG. 4. Deduced amino acid sequences of cDNAs encoding E4TF1-60 and -53. (A) Sequences of E4TF1-60 and GABP-α. (B) Sequences of E4TF1-53 and -47 and GABP-β. For the amino acid sequence of GABP, only different amino acids are shown. Stippled amino acids indicate those determined from purified proteins. Ets and notch homologous regions are indicated by the wavy lines. E4TF1-53- and -47-specific amino acids are indicated by dashed underlines. Asterisk indicates the position of the valinyl residue insertion that was not found in one cDNA clone of E4TF1-47.

and this difference correlates well with that of E4TF1-53 and E4TF1-47 as estimated by SDS-PAGE. Although amino acid sequence data were not obtained from purified E4TF1-47, we tentatively assumed that the 383-residue polypeptide was E4TF1-53 and that the 347 residue polypeptides was E4TF1-47.

Characterization of prokaryotic expressed E4TF1. To confirm that the obtained cDNA clones were subunits of E4TF1, they were expressed in *E. coli* and DNA binding activity and protein-protein interactions were examined. Open reading frames from each clone were introduced into the expression vector pET3d and expressed in *E. coli* BL21 (DE3) as described in Materials and Methods. The total extract was fractionated by SDS-PAGE, and each subunit was excised from the gel and renatured. The renatured proteins were examined for the ability to bind to DNA or the DNA-protein complex. As shown in Fig. 5A (lane 1), recombinant E4TF1-60 bound to the E4TF1-specific DNA with a mobility that was exactly the same as that of affinity-purified E4TF1-60. This retarded band was inhibited only when a specific competitor was added in the binding reaction (data not shown).

The recombinant E4TF1-53 and E4TF1-47 did not show any DNA binding activity by themselves, as expected from the purified protein (Fig. 5A, lanes 2 and 3). When recombinant E4TF1-53 was incubated with recombinant E4TF1-60 and the DNA probe, two more retarded bands, shown as complexes II and III in Fig. 2C, emerged (Fig. 5B, lanes 4 to 6). The positions of the complexes were the same as those formed with affinity-purified E4TF1-53 (Fig. 5B, lane 7).

Recombinant E4TF1-47 was also analyzed by means of gel retardation. As shown in Fig. 5B, a retarded band, whose mobility was the same as that of purified E4TF1-47, was observed when added to the mixture of E4TF1-60 and the DNA probe. This recombinant protein had same molecular mass as the lower band of E4TF1-47 on SDS-PAGE (data not shown). Moreover, the recombinant protein cross-reacted with GABP β, as did affinity-purified E4TF1-47 (data

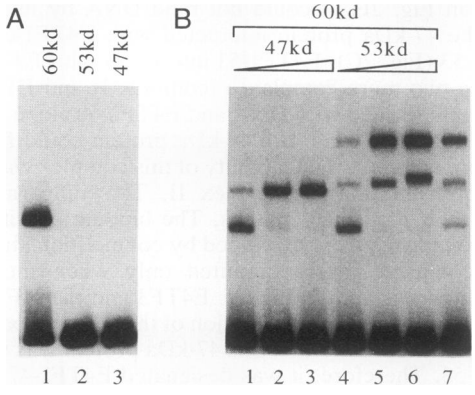

FIG. 5. DNA binding activity of recombinant E4TF1-60, -53, and -47. (A) Bacterial recombinants E4TF1-60 (lane 1), -53 (lane 2), and -47 (lane 3) were examined for their DNA binding activities. The proteins were prepared as described in Materials and Methods, and 1 μl of each protein was used. (B) Complex formation ability of recombinant E4TF1. Various amounts of recombinant E4TF1-53 (lanes 4 to 6) or -47 (lanes 1 to 3) were added to the gel retardation assay, which contains constant amount of E4TF1-60. Triangles show increasing amounts of E4TF1-47 and -53. Lanes 1 to 6, 1 μl of E4TF1-60; lanes 1 to 3 and lanes 4 to 6, 0.01, 0.1, and 1.0 μl of E4TF1-47 and E4TF1-53, respectively, was added. Lane 7 contained 0.5 μl of affinity-purified E4TF1.

not shown). These results confirmed that the cloned cDNAs encode E4TF1-60, E4TF1-53, and E4TF1-47.

E4TF1 genes are highly conserved in humans and rats. Computer analysis of the nucleotide sequence of the open reading frame revealed that E4TF1-60 and E4TF1-53 had very high homology with rat GABP-α (89.7%) and GABP-β1 (91.5%), respectively. GABP was identified as a DNA binding protein which recognizes the hexanucleotide sequence at the immediate-early *cis*-regulatory sequence ICP4 promoter of herpes simplex virus type 1 (13, 14). Comparison of the amino acid sequences showed 97.6 and 97.9% homology with GABP-α and -β 1 sequences, respectively. E4TF1-47 also showed high homology to GABP-β2. This suggested that E4TF1-60, E4TF1-53, and E4TF1-47 are human homologs of GABP-α, GABP-β1, and GABP-β2, respectively. Thus, we tested whether antisera to GABP-α and -β would cross-react with the corresponding subunit of E4TF1. Affinity-purified E4TF1 was Western blotted (immunoblotted), and antisera to GABP-α cross-reacted with E4TF1-60, whereas antisera to GABP-β reacted with both E4TF1-53 and -47 (Fig. 6A). These antisera also cross-reacted with recombinant E4TF1 subunit proteins. The cross-reaction was further confirmed by gel retardation with these antisera. Complex I, formed with E4TF1-60 and the DNA probe, was supershifted when anti-GABP-α, but not anti-GABP-β, was added (Fig. 6B, lanes 2 and 3). The low mobility of the heterodimer complex, complex II and III, composed of E4TF1-60 and -53 and DNA was supershifted by the addition of either anti-GABP-α or anti-GABP-β Fig. 6B, lanes 6 and 7). Similar supershifts were performed to analyze the complex formed with E4TF1-47. The slow mobility complex was supershifted by both anti-GABP-α and anti-GABP-β (Fig. 6B, lanes 10 and 11). When anti-GABP-β was added to the reaction mixture, only the slow-mobility complex was supershifted. We confirmed that the slow-mobility complex was composed of E4TF1-47 and -60 and the DNA probe. These observations supported the notion that E4TF1-47 interacts with E4TF1-60. Sequence homologies between E4TF1 and GABP and cross-reaction with antisera indicated that E4TF1 is the human homolog of rat GABP.

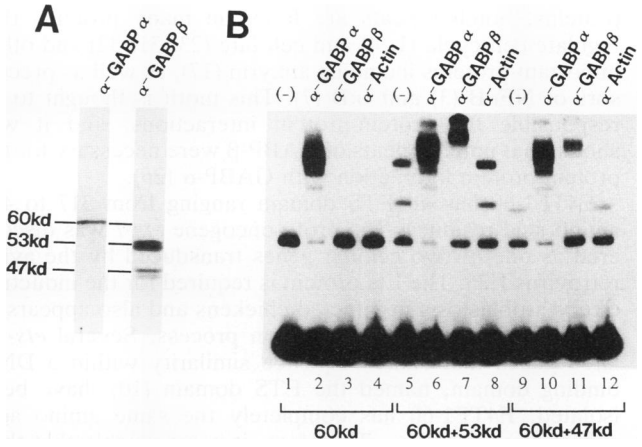

FIG. 6. Immunological cross-reaction of E4TF1 and GABP. (A) Western blots of E4TF1 with anti-GABP-α (lane 1) and -β (lane 2). Affinity latex-purified E4TF1 (4.0 μl) was separated by SDS-PAGE and Western blotted as described in Materials and Methods. (B) Antibody against GABP-α or -β was added in a gel retardation assay which contains renatured 60-kDa (lanes 1 to 4), 60- and 53-kDa (lanes 5 to 8), or 60- and 47-kDa (lanes 9 to 12) proteins. The added antibodies are indicated at the top. Antibody against actin (α-Actin) was added as a control. One microliter of 60- and 53-kDa protein and 2 μl of 47-kDa protein were used in the reaction.

DISCUSSION

We originally identified E4TF1, which regulates transcription from the adenovirus E4 promoter, by fractionating and reconstituting HeLa cell nuclear extracts (29). We characterized E4TF1 which was purified from the enriched fraction and found that it is composed of two subunits, E4TF1-60 and E4TF1-53 (30). We recently developed a method of using affinity latex particles to quickly and efficiently purify DNA binding proteins directly from crude nuclear extracts without fractionation (8). The availability of purified E4TF1 enabled determination of the partial amino acid sequences of E4TF1-60 and E4TF1-53 and also identification of another protein, E4TF1-47, which interacted with E4TF1-60. On the basis of these sequences, we isolated their cDNA clones from a HeLa cell cDNA library.

Affinity-purified E4TF1 was analyzed by SDS-PAGE (Fig. 2). Three major polypeptides with double bands, shown as 60, 53, and 47 kDa, were copurified. To characterize each polypeptide, we denatured and renatured them and then assayed their activities. The upper 60-kDa band bound to DNA, but the lower band did not (data not shown). Antiserum to GABP-α cross-reacted only with the upper band (Fig. 6A). Furthermore, the recombinant E4TF1 showed only a single band by SDS-PAGE, the position of which was the same as the upper band (data not shown). The results indicated that E4TF1-60 corresponded to the upper band. We have not yet characterized the lower band. As for the 53- and 47-kDa proteins, antiserum to GABP-β cross-reacted with two bands for each of the 53- and 47-kDa proteins (Fig. 6A). The positions of recombinant E4TF1-53 and E4TF1-47 by SDS-PAGE corresponded with the lower band of each. This was also confirmed by HPLC fractionation after digestion of each E4TF1-53 band with lysyl-endopeptidase. The fractionated peaks of the upper and lower bands of E4TF1-53 exhibited the same profile, except for one peak. Therefore, the appearance of two bands for E4TF1-53 and E4TF1-47 was derived from posttranslational modifications, although the exact reasons remain unknown. Other two minor bands whose molecular masses were 92 and 65 kDa were able to bind to the E4TF1 site but not to interact with E4TF1-53 (data not shown).

Affinity-purified and recombinant E4TF1 subunits showed almost the same complex formation abilities. Two complexes (complexes II and III) were found when E4TF1-53 was added to E4TF1-60 in a gel retardation assay (Fig. 2 and 5). On the other hand, only one complex was found when E4TF1-47 was added. This might be due to the differences in their C-terminal sequences. E4TF1-53 and E4TF1-47 had a common N-terminal sequence spread over 332 amino acids but different C-terminal sequences. In the C-terminal region of E4TF1-53, there were 21 positively and negatively charged amino acid residues. On the other hand, there were four positively charged amino acids at the E4TF1-47-specific C terminus. Thompson et al. (26) reported that GABP-β1, which has an amino acid sequence very similar to that of E4TF1-53, could form a homodimer, and its C terminus was essential for this interaction.

From the amino acid sequences predicted from the cDNA, typical motifs were found in the E4TF1 subunit. At the N termini of E4TF1-53 and E4TF1-47, four imperfect tandem repeats of 33 amino acids were observed, and the 33-amino-acid sequence was similar to that repeated in notch-related

proteins. notch repeats are found in many proteins that regulate cell cycle (1, 4) and cell fate (23, 31, 32) and other important proteins including ankyrin (17), as well as precursors of NFκB (3) and IκB (7). This motif is thought to be responsible for protein-protein interactions, and it was shown that notch repeats of GABP-β were necessary for the protein-protein interaction with GABP-α (26).

E4TF1-60 has an ETS domain ranging from 317 to 401 amino acid residues. The proto-oncogene *ets-1* was discovered as one of two cellular genes transduced by the avian retrovirus E26. The Ets protein is required for the induction of erythroblastosis in infected chickens and also appears to affect the myeloid transformation process. Several *ets*-related genes that share sequence similarity within a DNA binding domain, named the ETS domain (10), have been isolated. E4TF1-60 has completely the same amino acid sequence as GABP-α. Therefore, it is reasonable that they both recognize the same DNA sequence.

E4TF1 and GABP sites are located in the region responsible for the viral transactivating factors, E1A and VP16, respectively. In the adenovirus E4 promoter region, there are two distinct *cis*-regulating elements that are responsible for transactivation of E1A. One is the E4TF1/ATF site. This element is found at least at four sites in the E4 promoter. The other is an E4TF1 binding site. In the promoter region of the ICP4 (27), which is an immediate-early gene of herpes simplex virus type 1, there are two distinct *cis*-regulatory motifs that mediate *trans*-activation by VP16. One motif is the nonanucleotide sequence 5′-TAATGARAT-3′ (R = purine), and it is recognized by Oct-1. This motif is found at three sites in the ICP4 promoter. VP16 does not bind directly to either of the *cis*-regulatory sequences and functions in a complex with the cellular transcription factor Oct-1. The other motif is the purine-rich hexanucleotide sequence 5′-GCGGAA-3′, and GABP recognizes this hexanucleotide sequence.

E1A protein and VP16 protein are viral transactivators. Neither of them bind to specific DNA, and it is speculated that they function through protein-protein interactions (18). It has been reported that E1A and VP16 activates transcription via DNA-binding proteins, E4TF3/ATF and Oct-1, respectively (16, 24). Both E1A and VP16 have an acidic region which is responsible for transactivation, and the N terminus E1A acidic region can be replaced by that of VP16 at the C terminus (15). The binding sites of E4TF1 and GABP are located within the responsible regions for these viral transactivators and are adjacent to the E4TF3/ATF and Oct-1 sites, respectively. This suggests that E4TF1 and GABP, in addition to E4TF3/ATF and Oct-1, play an important role in transactivation by viral proteins.

We have reported that E4TF1-53 conferred transcription stimulation activity upon E4TF1-60. Therefore, it would be of interest to know with which proteins responsible for activation of transcription from the E4 promoter E4TF1-53 interacts.

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