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Transcriptional activation through the tetrameric complex formation of E4TF1 subunits

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Transcription factor E4TF1 is composed of two types of subunit, an ets-related DNA binding protein, E4TF1-60, and its associated proteins with four tandemly repeated Notch-ankyrin motifs, E4TF1-53 and E4TF1-47. To determine the functional domains, we constructed various mutants of the subunits. E4TF1-60 bound to DNA as a monomer. The ets domain and its N-terminal flanking region were necessary to recognize the specific DNA sequence. The 48 amino acids at the E4TF1-60 C-terminus were required for interaction with the other type of subunit. E4TF1-53 and E4TF1-47 share the N-terminal 332 amino acids but differ at the C-termini. They interacted with E4TF1-60 through the N-terminal flanking region to form a heterodimer. E4TF1-53 dimerized with itself, whereas E4TF1-47 did not. The C-terminal region specific for E4TF1-53 was required for the dimerization. Therefore, heterodimers composed of E4TF1-53 and E4TF1-60 were further dimerized, resulting in the formation of a tetrameric complex, which stimulated transcription *in vitro*. Heterodimers of E4TF1-47 and E4TF1-60 weakly stimulated transcription *in vitro*. The results indicated that the tetrameric complex formation of E4TF1 subunits was necessary to activate transcription efficiently *in vitro*.

Key words: E4TF1/heterodimer/tetramer/transcriptional activation/transcription factor

Introduction

Transcription initiation in eukaryotes is regulated by various *trans*-acting factors including general and gene-specific transcription factors. Gene-specific transcriptional factors bind to *cis*-acting elements of the gene and stimulate the basal level of transcription mediated by general transcription factors. Protein–protein interactions between the gene-specific and general transcription factors are important mechanisms of transcriptional activation. Likewise, interactions between proteins are important for the DNA binding and activating properties of gene-specific transcription factors, such as ATF (Lee *et al.*, 1987; Hai *et al.*, 1988; Wada *et al.*, 1991), Ap-1 (Chiu *et al.*, 1988; Curran and Franza, 1988) and MyoD (Murre *et al.*, 1989a,b; Davis *et al.*, 1990). These factors function as homo- and hetero-dimers formed with related factors through basic helix–loop–helix or leucine zipper motifs (Jones, 1990).

We previously reported that the transcription factor, E4TF1, consisted of at least two distinct factors with molecular masses of 60 and 53 kDa, designated E4TF1-60 and E4TF1-53, respectively (Watanabe *et al.*, 1988, 1990). E4TF1 was originally identified as one of the factors responsible for transcription from the adenovirus early region 4 (E4) promoter (Watanabe *et al.*, 1988). We recently isolated the cDNAs of the factors and identified a new E4TF1 member with a molecular mass of 47 kDa, designated E4TF1-47 (Watanabe *et al.*, 1993). The sequence data have shown that E4TF1-60, E4TF1-53 and E4TF1-47 are highly homologous to GABPα, GABPβ1 and GABPβ2 (LaMarco *et al.*, 1991), respectively. Therefore, we believe that E4TF1 is a human homolog of rat GABP, which has been purified as a factor that recognizes the *cis*-acting elements of herpes simplex virus immediate early genes (LaMarco *et al.*, 1989; Thompson *et al.*, 1991). Recently, some transcription factors, EF-1A (Bolwig *et al.*, 1992), NRF-2 (Virbasius *et al.*, 1993) and β-factor (Yoganathan *et al.*, 1992), responsible for transcription of the adenovirus early region 1a, the rat cytochrome *c* oxidase subunit IV gene and mouse ribosomal protein L32 gene, respectively, have been found to be related to GABP.

E4TF1-60 alone can bind to a specific DNA sequence but does not activate transcription *in vitro* (Watanabe *et al.*, 1990). E4TF1-53 alone neither binds to DNA nor stimulates transcription *in vitro*. However, E4TF1-53 and E4TF1-47 interact with E4TF1-60, which has an ets DNA binding domain (Watanabe *et al.*, 1993). The interaction of E4TF1-53 with E4TF1-60 confers the ability to stimulate transcription *in vitro* (Watanabe *et al.*, 1990). E4TF1-47 has the same 332 amino acid residues at the N-terminus as E4TF1-53. Four tandemly repeated Notch-ankyrin motifs are present at the N-terminus. E4TF1-53 and E4TF1-47 have distinct 50 and 15 amino acid residues, respectively, at their C-termini. This distinction might be involved in their activities, because they exhibit different complex formation in association with E4TF1-60 in gel shift assays. When E4TF1-53 is mixed with E4TF1-60, two more slowly migrating complexes appear in addition to that complex composed of E4TF1-60 and the probe. However, only one more complex was formed with E4TF1-47 (Watanabe *et al.*, 1993). These results indicate that the E4TF1 subunits have multiple functions. To elucidate the functional domains of each E4TF1 subunit, we constructed several deletion mutants of E4TF1-60 and E4TF1-53 and examined their interactions with either DNA or other subunits and their ability to stimulate transcription *in vitro*.

In this study, we identified the E4TF1-60 domains that are responsible for binding to DNA and for interacting with E4TF1-53 and also the E4TF1-53 domains that are involved in interaction with E4TF1-60 and dimerization. E4TF1-60 formed a heterodimer with either E4TF1-53 or E4TF1-47. The heterodimer of E4TF1-53 was further dimerized, resulting in a tetrameric complex, which may function as

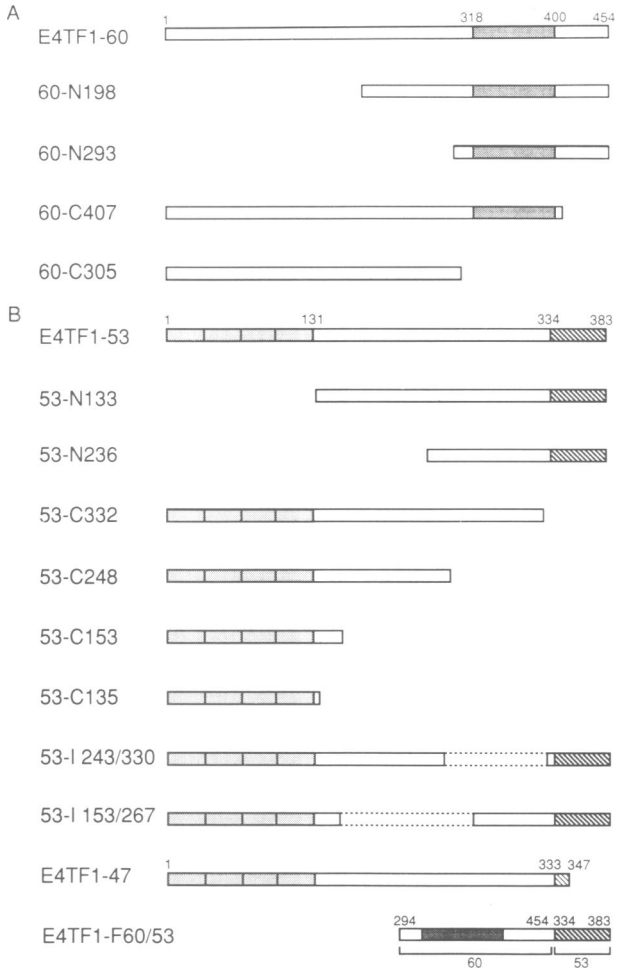

Fig. 1. Schematic structures of full-length and truncated E4TF1 subunits. (**A**) E4TF1-60 and its deletion mutants. Stippled regions indicate the ets-related domain. (**B**) E4TF1-53 and its mutants. Stippled regions indicate four tandem repeats of the Notch-ankyrin motif. Hatched regions at the C-terminus indicate the E4TF1-53-specific region lacking in E4TF1-47.

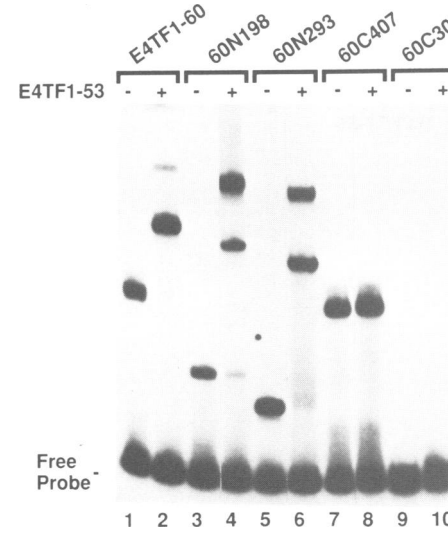

Fig. 2. The ability of E4TF1-60 and its mutants to bind to DNA and interact with E4TF1-53. Gel shift assays were performed using 0.2 μl of each recombinant protein. To test their ability to interact with E4TF1-53, 0.2 μl of recombinant E4TF1-53 was added to the binding reaction (lanes 2, 4, 6, 8 and 10).

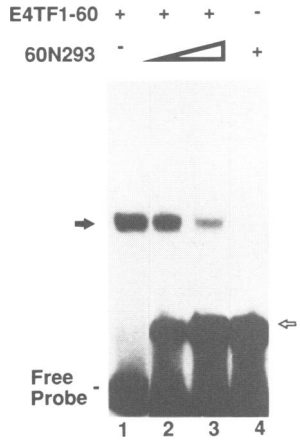

Fig. 3. E4TF1-60 binds to DNA as a monomeric form. Gel shift assays were performed as described (Watanabe *et al.*, 1988). The binding reaction contained 0.2 μl of E4TF1-60 (lane 1), 0.2 μl of E4TF1-60 and 0.02 μl of 60-N293 (lane 2), 0.2 μl of E4TF1-60 and 0.2 μl of 60-N293 (lane 3), and 0.2 μl of 60-N293 (lane 4).

a mediator between two DNA molecules. The tetramer efficiently activated transcription *in vitro*. On the other hand, the E4TF1-47 heterodimer, lacking dimerization activity, did not efficiently activate transcription *in vitro*. These results indicated that the formation of a tetrameric complex of E4TF1 was required to activate transcription *in vitro*.

Results

Dissection of functional domains of E4TF1-60

We previously described that E4TF1-60 has at least two regions that bound to the specific DNA sequence and interacted with E4TF1-53. The interaction was necessary for E4TF1 to function as a transcription factor. To identify the functional domains of E4TF1-60, a series of deletion mutants was constructed (Figure 1) and expressed in *Escherichia coli* using a T7 polymerase-dependent expression system (Studier *et al.*, 1990). All the recombinant proteins were fractionated by SDS−PAGE, eluted from the gel, then denatured and renatured as previously described (Watanabe *et al.*, 1990).

Their DNA binding activity and E4TF1-53 interactions were examined by gel shift assays. Figure 2 shows that the C-terminal deletion mutant, 60-C305, lost DNA binding activity. Since 60-C407 bound to DNA, the region homologous to ets-related domain was required for DNA binding.

Figure 2 also shows the domains of E4TF1-60 responsible for interaction with E4TF1-53. When E4TF1-53 interacted with E4TF1-60, two more slowly migrating complexes were formed in addition to that composed of E4TF1-60 and the DNA probe (lanes 1 and 2). Two more slowly migrating complexes were also formed when 60-N198 and 60-N293 were used (lanes 4 and 6), indicating that the mutants interacted with E4TF1-53. However, 60-C407 did not form a slowly migrating complex, indicating that the C-terminus of E4TF1-60, adjacent to the ets domain, was necessary to interact with E4TF1-53.

DNA binding of E4TF1-60 as a monomeric form

The positions of complexes formed with E4TF1-60 and 60-N293 were apparently different according to a gel shift

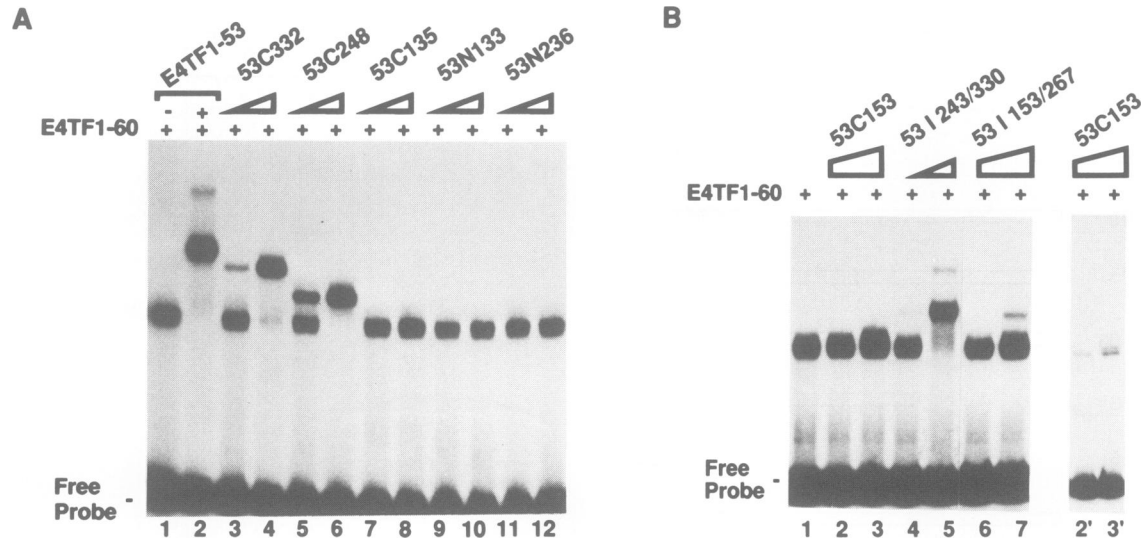

Fig. 4. The interaction domain of E4TF1-53 with E4TF1-60. (**A**) Gel shift assays were performed using E4TF1-60 and mutants of E4TF1-53 as described (Watanabe *et al.*, 1988). Various amounts of E4TF1-53 mutants were added to the binding reaction containing a constant amount (0.2 μl) of E4TF1-60 (lanes 1−12). The reaction contained 0.02 μl (lanes 3, 5, 7, 9 and 11) and 0.2 μl (lanes 4, 6, 8, 10 and 12) of each mutant protein. Control experiments in the absence (lane 1) and the presence of 0.2 μl of E4TF1-53 (lane 2) were performed. (**B**) Gel shift assays included internal deletion mutants of E4TF1-53. The reaction contained 0.01 μl (lane 4), 0.1 μl (lanes 2, 5 and 6) and 1.0 μl (lanes 3 and 7) of the indicated protein in the presence of a constant amount of E4TF1-60 (0.2 μl). The complex formed with E4TF1-60 and the DNA probe is indicated in lane 1. The increase in each protein level is shown as a triangle and trapezoids. Lanes 2′ and 3′ are the short exposure of lanes 2 and 3, respectively.

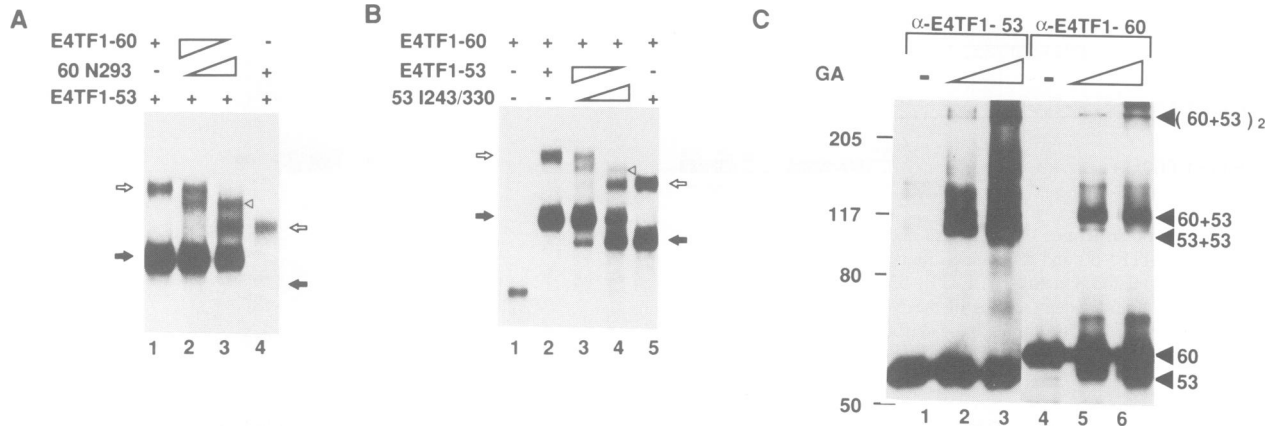

Fig. 5. Tetramer complex formation of E4TF1. (**A**) E4TF1-60 and 60-N293 were mixed with E4TF1-53 in the binding reaction and gel shift assays were performed as described (Watanabe *et al.*, 1988). Lane 1, 0.2 μl of E4TF1-60 and 0.4 μl of E4TF1-53; lane 2, 0.15 μl of E4TF1-60, 0.4 μl of E4TF1-53 and 0.05 μl of 60-N293; lane 3, 0.1 μl of E4TF1-60, 0.4 μl of E4TF1-53 and 0.1 μl of 60-N293; lane 4, 0.2 μl of 60-N293 and 0.4 μl of E4TF1-53. (**B**) E4TF1-53 and 53-I243/330 were mixed with E4TF1-60 in the binding reaction. Lane 1, 0.2 μl of E4TF1-60; lane 2, 0.2 μl of E4TF1-60 and 1.0 μl of E4TF1-53; lane 3, 0.2 μl of E4TF1-60, 1.0 μl of E4TF1-53 and 0.5 μl of 53-I243/330; lane 4, 0.2 μl of E4TF1-60, 0.5 μl of E4TF1-53 and 1.0 μl of 53-I243/330; lane 5, 0.2 μl of E4TF1-60 and 1.0 μl of 53-I243/330. Intermediate complexes are indicated as open triangles. Closed and open arrows indicate complexes composed of the corresponding heterodimers and tetramers respectively. The heterodimer composed of 60-N293 and E4TF1-53 was not observed, because the tetramer was easily formed under these conditions. (**C**) Chemical cross-linking of E4TF1. E4TF1-60 and E4TF1-53 (120 ng each) were incubated for 30 min at 30°C in a buffer containing 30 mM HEPES−NaOH (pH 7.9), 12% glycerol, 30 mM KCl and 0.6 mM DTT. Then, varying concentrations of glutaraldehyde (GA) (lanes 2 and 5, 0.0005%; lanes 3 and 6, 0.0015%) were added to the binding reaction and incubated for 30 min at 30°C. The samples were then subjected to electrophoresis on a denaturing 8% polyacrylamide gel and transferred to a membrane. The membrane was blocked with 5% non-fat dried milk in TBS, 0.05% Tween-20 for 2 h and incubated with antibodies against either E4TF1-53 (lanes 1−3) or E4TF1-60 (lanes 4−6) for 8 h, followed by secondary antibodies conjugated to horseradish peroxidase, which was visualized using hydrogen peroxide and diaminobenzidine (Watanabe *et al.*, 1993).

assay (see Figure 2). We therefore examined whether E4TF1-60 bound to DNA as a monomer or as a dimer. We mixed E4TF1-60 and 60-N293 with the DNA probe in the binding reaction and performed a gel shift assay using a DNA probe that contained a single E4TF1 binding site. If they bound to DNA as a dimer, a protein−DNA complex formed by E4TF1-60 and 60-N293 would have intermediate mobility in the gel shift assay compared with homotypic complexes composed solely of E4TF1-60 and 60-N293. Figure 3 shows that such a complex was not detected, indicating that E4TF1-60 bound to DNA as a monomer.

Dissection of the functional domains of E4TF1-53

To determine the functional domains of E4TF1-53, we prepared recombinant proteins with deletions (see Figure 1). The proteins were incubated with E4TF1-60 and the DNA probe in the binding reaction and their ability to interact with E4TF1-60 was analyzed by gel shift assays. As shown in

Figure 4A, two N-terminal deletion mutants, 53-N133 and 53-N236, did not interact with E4TF1-60, indicating that the E4TF1-53 N-terminal region was necessary for interaction with E4TF1-60. The mutant, 53-C135, which contained all the Notch-ankyrin repeats did not interact with E4TF1-60. Mutants 53-C332 and 53-C248 did interact with E4TF1-60 but formed only one slowly migrating complex. Figure 4B shows that 53-I243/330 interacted with E4TF1-60, indicating that the region between 243 and 330 amino acid residues was not necessary. Though 53-C153 bound to E4TF1-60, the activity was lower than those of 53-C248 and 53-C332 (Figure 4A and B). As compared with 53-C332, ~10 times more 53-C153 was necessary to form the same amount of complex with E4TF1-60. An internal deletion mutant, 53-I153/267, had the same activity level as 53-C153. These results indicated that, in addition to Notch-ankyrin repeats, the C-terminal flanking region was required for interaction of E4TF1-53 with E4TF1-60.

One issue was the formation of the two slowly migrating complexes in gel shift assays when E4TF1-60 was mixed with E4TF1-53. Since E4TF1-60 bound to DNA as a monomer (see Figure 3), we assumed that the most slowly migrating complex was derived from dimerization of heterodimers formed with E4TF1-60 and E4TF1-53. If so, E4TF1-53 would contain the domain required for dimerization. The C-terminal deletion mutants, 53-C332 and 53-C248, did not form the top complex (Figure 4A, lanes 4 and 6). By adding the C-terminal region to 53-C242, this activity was recovered as shown in 53-I243/330 (Figure 4B, lane 5). This suggested that the C-terminal 52 amino acid residues were involved in dimerization of E4TF1-53. Chemical cross-linking revealed that E4TF1-53 formed a homodomer (data not shown). These results suggested that heterodimers composed of E4TF1-60 and E4TF1-53 could form a tetramer through E4TF1-53 dimerization. Involvement of the C-terminus specific for E4TF1-53 in dimerization was also supported by our previous results which showed that a variant of E4TF1-53, E4TF1-47, that contained C-terminal amino acid residues different from E4TF1-53 could not form the most slowly migrating complex (Watanabe *et al.*, 1993). These results suggested that E4TF1-60 and E4TF1-53 formed a heterodimer which was further dimerized through the E4TF1-53 C-terminus, resulting in a tetramer.

Tetrameric complex formation of E4TF1-60 and E4TF1-53

To confirm the formation of a tetrameric complex, E4TF1-53 was mixed with both E4TF1-60 and 60-N293 in the binding reaction and complex formation was analyzed by gel shift assays. Figure 5A shows that an intermediate complex was formed between the two most slowly migrating complexes formed with E4TF1-53 and either E4TF1-60 or 60-N293. Moreover, an additional intermediate complex was also detected between the two top complexes composed of E4TF1-60 and either E4TF1-53 or 53-I243/330, when E4TF1-60 was mixed with both E4TF1-53 and 53-I243/330 in the binding reaction (Figure 5B). These results confirmed that the most slowly migrating complex was composed of a tetramer formed by two molecules each of E4TF1-60 and E4TF1-53.

Furthermore, the results indicated that the tetrameric complex formed by E4TF1-60 and E4TF1-53 mediated the interaction between two DNA molecules, because our DNA probe contained a single E4TF1 binding site.

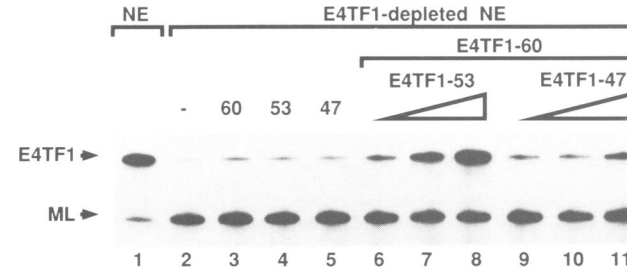

Fig. 6. A comparison of the *in vitro* transcription stimulating activities of recombinant E4TF1-53 and E4TF1-47. The reaction contained the indicated E4TF1 subunits. One microliter of E4TF1-60 (lane 3), 1.0 μl of E4TF1-53 (lane 4) and 1.0 μl of E4TF1-47 (lane 5) were added to the reaction; 0.1 μl (lane 6), 0.3 μl (lane 7) and 1.0 μl (lane 8) of E4TF1-53 were mixed with 1.0 μl of E4TF1-60. 0.1 μl (lane 9), 0.3 μl (lane 10) and 1.0 μl (lane 11) of E4TF1-47 were mixed with 1.0 μl of E4TF1-60. The basal transcriptional activity of the E4TF1-depleted nuclear extracts is shown in lane 2. NE indicates the original crude nuclear extracts without E4TF1 depletion. E4TF1 and ML indicate the products transcribed from the promoter containing the E4TF1 binding sites (Watanabe *et al.*, 1990) and the adenovirus major late promoter, respectively. These promoters have G-free cassettes of various lengths as described (Watanabe *et al.*, 1990).

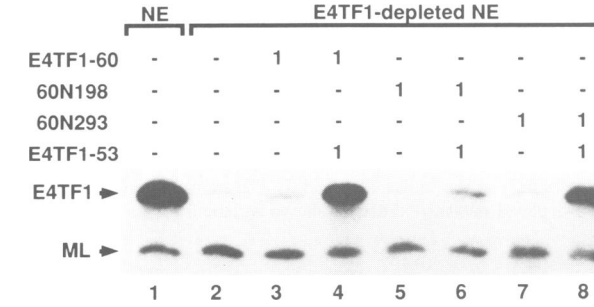

Fig. 7. Transcription stimulating activities of E4TF1-60 mutants. Recombinant proteins, 1.0 μl each, were added to the reaction in the absence (lanes 2, 3, 5 and 7) or the presence (lanes 4, 6 and 8) of E4TF1-53. NE indicates the crude nuclear extracts without E4TF1 depletion.

The existence of a tetrameric complex was further confirmed by chemical cross-linking experiments. In the presence of glutaraldehyde, the bands with the molecular weights of ~110 and 220 kDa were reacted with both antibodies against E4TF1-60 and E4TF1-53. The result indicated that they corresponded to dimeric and tetrameric complexes formed with E4TF1-60 and E4TF1-53.

Analysis of the transcription activation domain of E4TF1-60 and E4TF1-53

E4TF1 was originally identified as a DNA binding transcription factor that stimulated the *in vitro* transcription of adenovirus early region 4 (Watanabe *et al.*, 1988). We previously showed that the interaction between both subunits, E4TF1-60 and E4TF1-53, are required for transcriptional activation (Watanabe *et al.*, 1988). The transcription stimulation activity of the recombinant E4TF1 subunits was determined using *in vitro* transcription assays described previously (Watanabe *et al.*, 1990). Figure 6 shows that neither protein alone stimulated transcription from either the E4 promoter or the major late promoter (MLP) of adenovirus. When E4TF1-53 was mixed with E4TF1-60, transcription from the E4 was selectively activated but not from the MLP used as an internal control. On the other hand,

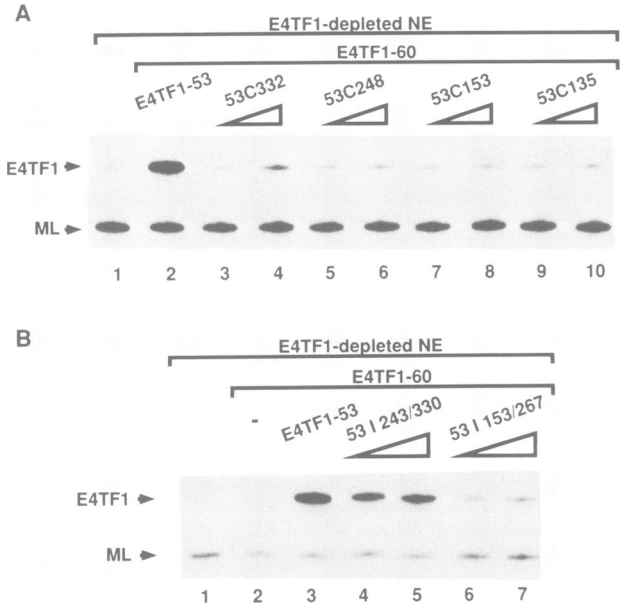

Fig. 8. Transcription stimulating activities of E4TF1-53 mutants. (**A**) E4TF1-53, 1.0 μl, and different quantities of its C-terminal deletion mutants were mixed with 1.0 μl of E4TF1-60 in the reaction (lanes 2–10). The reaction contained 1.0 μl (lanes 3, 5, 7 and 9) and 10 μl (lanes 4, 6, 8 and 10) of the indicated proteins. The basal transcriptional activity using E4TF1-depleted extracts is shown in lane 1. (**B**) E4TF1-53, 1.0 μl (lane 3) and the indicated mutant proteins were added to the transcription reaction containing 1.0 μl of E4TF1-60 (lanes 2–7). The reaction included 1.0 μl (lanes 4 and 6) and 10 μl (lanes 5 and 7) of the indicated proteins. The transcriptional activity of E4TF1-depleted nuclear extracts is shown in lane 1.

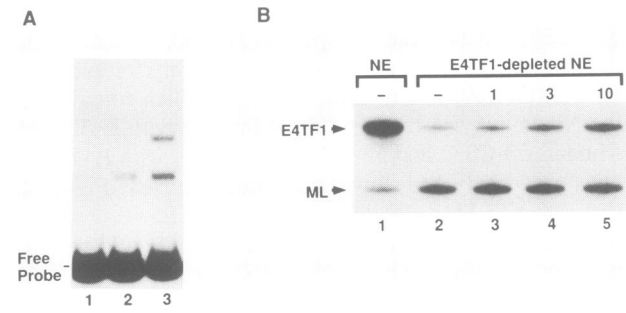

Fig. 9. Transcription stimulation activities of E4TF1-F60/53. (**A**) Gel shift assay of E4TF1-F60/53. The reaction contained 0.5 ng (lane 1), 1.7 ng (lane 2) and 5 ng (lane 3) of E4TF1-F60/53. (**B**) Indicated volumes (μl) of E4TF1-F60/53 (5 ng/μl) were added to the transcription reaction.

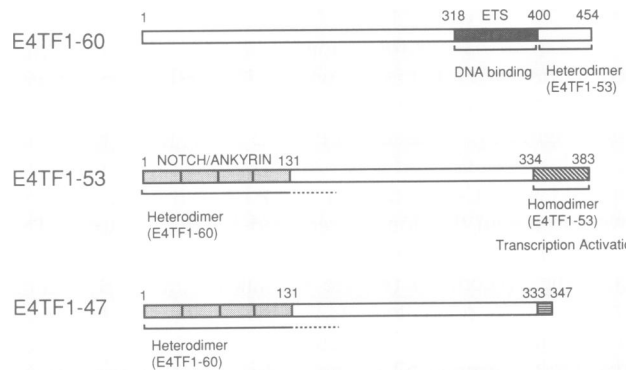

Fig. 10. Summary of the functional domain of E4TF1.

E4TF1-47 did not activate E4 transcription as efficiently as E4TF1-53 (lanes 9–11). The activity of E4TF1-47 was about one-tenth that of E4TF1-53.

To study further the relationship between complex formation and transcriptional activation, mutant E4TF1-60 and E4TF1-53 proteins were tested using *in vitro* transcription assays. Figure 7 shows that 60-N293 activated the E4 transcription as efficiently as E4TF1-60. This indicated that the E4TF1-60 C-terminal region which bound to DNA and interacted with E4TF1-53 was required for transcription stimulation *in vitro* and that the N-terminal 293 amino acid residues of E4TF1-60 were not necessary for activation. The mutant 60-N198 repeatedly had less activity than 60-N293 in several trials. It is possible that the region between amino acid residues 199 and 293 contained an inhibitory element for transcription activation. If so, the N-terminal 198 amino acid residues would block the inhibitory effect.

We also analyzed the transcription stimulation activities of the E4TF1-53 mutants. Figure 8A shows that none of the C-terminal deletion mutants, except 53-C332, activated transcription. It was slightly stimulated by increasing the amount of 53-C332 (lane 4). The N-terminal deletion mutants, 52-N133 and 53-N236, did not stimulate transcription (data not shown). These mutants could not form a tetrameric complex (see Figure 4A). To analyze further the relationship between the complex formation and transcriptional activation, internal deletion mutants were tested. Figure 8B shows that 53-I243/330 activated E4 transcription (lanes 4 and 5). The mutant 53-I153/267, which hardly interacted with E4TF1-60 (see Figure 4B), had very weak activity (lanes 6 and 7). These results indicated that the formation of a stable tetrameric complex composed of two molecules each of E4TF1-60 and E4TF1-53 was required for efficient activation of *in vitro* transcription.

To analyze further whether the C-terminal region of the 53 kDa subunit is involved in transcriptional activation, we constructed a chimeric protein, E4TF1-F60/53, consisting of the ets DNA binding domain of E4TF1-60 and the C-terminus of E4TF1-53. The gel shift experiment using this chimeric protein showed that it bound to E4TF1-specific DNA sequence and it formed homodimer in a dose-dependent manner (Figure 9A). These complexes were competed out when E4TF1-specific DNA was added in the gel shift assay as a competitor (data not shown). By using an *in vitro* transcription assay, the chimeric protein specifically activated transcription from the promoter-containing E4TF1 binding site.

Discussion

We demonstrated here that a heterodimer formed with E4TF1-60 and E4TF1-53 bound to an E4TF1 binding site and that the heterodimers were further dimerized, resulting in a tetramer that efficiently activated E4 transcription *in vitro*. These domains necessary for protein–protein interaction and transcription activation are summarized in Figure 10. One important finding of this study was the difference between E4TF1-53 and E4TF1-47 with regard to transcription stimulation. E4TF1-53 and E4TF1-47 have the same N-terminal amino acid sequences but differ at their C-termini. Fifty amino acid residues of the C-terminus are characteristic of E4TF1-53. We showed that this region was

essential for tetramer formation and for efficient transcription activation. On the other hand, E4TF1-47 lacking the sequence did not form a tetramer. E4TF1-47 did not activate transcription *in vitro* as efficiently as E4TF1-53. Fifteen amino acids of the C-terminal region specific for E4TF1-47 did not function as an inhibitor of tetramer formation, because 53-C332, the C-terminal deletion mutant of E4TF1-53, could not form a tetramer. Thus, the C-terminal region specific for E4TF1-53 was necessary both for tetramer formation and for efficient transcription activation *in vitro*.

The function of the C-terminal region of E4TF1-53 was confirmed by using the internal deletion mutant, 53-I243/330, which interacted with both E4TF1-60 and E4TF1-53 (see Figure 5B) and efficiently activated transcription *in vitro* (see Figure 8B). Moreover, the chimeric protein that has the DNA binding domain of E4TF1-60 and the C-terminal region of E4TF1-53 functions as a transcription activator.

Both 53-C332 and E4TF1-47, which could not form a tetramer with E4TF1-60, had very weak but definite transcription stimulation activity at almost the same level. This suggested that the region between amino acid residues 248 and 331 of E4TF1-53, which is not necessary for tetramer, is involved in the activity (Figure 8A). Therefore, this region might be involved in weak transcription stimulation through a mechanism different from tetramer formation. Alternatively, E4TF1-47 might function as a repressor of the E4TF1-dependent transcription of competitively inhibiting the binding of E4TF1-53 to E4TF1-60. Further analyses are necessary to elucidate the functions of E4TF1-53 and E4TF1-47 in the regulation of transcription *in vivo*.

No known motif concerned with protein−protein interactions for dimerization was found in the E4TF1-53-specific C-terminal region. The region was rich in positively and negatively charged amino acid residues and the formation of an α-helix structure was predicted by the Chou−Fassman program. However, a precise mechanism was not identified. We are now further analyzing the sequence and structure essential for the dimerization by preparing other mutant proteins.

The N-terminal deletion mutants of E4TF1-53 showed that Notch-ankyrin repeats play an important role in the interaction between E4TF1-60 and E4TF1-53. Though the repeats are reportedly important for protein−protein interaction (Lux *et al.*, 1990; Thompson *et al.*, 1991), our results indicated that the repeats alone were not sufficient for the interaction (see Figure 4A and B). One of the mutants, 53-C135, which has the entire set of Notch-ankyrin repeats, did not bind to E4TF1-60. Moreover, 53-C153 interacted very weakly. The mutant 53-C248 interacted with E4TF1-60. These results indicated that the C-terminal flanking region, in addition to the Notch-ankyrin, was required for the interaction of E4TF1-53 with E4TF1-60. This observation was consistent with the study of Notch-ankyrin repeats in IκB. These repeats were required for protein−protein interactions but in themselves were insufficient for stable complex formation (Inoue *et al.*, 1992).

Our results indicated that the tetrameric complex formed with two molecules each of E4TF1-60 and E4TF1-53 mediated the intermolecular interaction between two DNA molecules (see Figure 5A and B). The DNA template used in *in vitro* transcription assays contained four E4TF1 binding sites at the promoter region. It was possible that two heterodimers of E4TF1-60 and E4TF1-53 coordinately interacted on one DNA molecule. However, the E4 wild type promoter contains only one E4TF1 binding site which was shown by DNase I footprinting described previously (Watanabe *et al.*, 1988, 1990). Therefore, E4TF1-60 and E4TF1-53 might activate transcription from the E4 promoter through the interaction between two DNA molecules. So far, dimerization of E4TF1-60 and E4TF1-53 heterodimers seems to be necessary for transcriptional activation *in vitro*. Further analyses are necessary to elucidate the relationship between the tetrameric complex formation and transcriptional activation. We are now trying to categorize these activities by further constructing more mutants lacking amino acid residues or by changing one residue in the C-terminal region specific for E4TF1-53.

Materials and methods

Construction of plasmids

To construct expression vectors of E4TF1-60 and E4TF1-53 deletion mutants, cDNAs of E4TF1-60 and E4TF1-53 were inserted into pET3d forming plasmids pET60 and pET53 (Watanabe *et al.*, 1993). To generate plasmids pET60-N198 and pET60-N298 expressing 60-N198 and 60-N293 (see Figure 1), *Eco*RI−*Bam*HI and *Hae*II−*Bam*HI fragments of pET60 were recloned into the *Nco*I−*Bam*HI site of pET3d. To introduce the first ATG codon at the beginning of their open reading frames, *Nco*I−*Eco*RI and *Nco*I−*Hae*II adaptors were synthesized and fused to the translation initiation sites, respectively. Their sequences were

5′- CATGGTTTGGGTAATAAAGG- 3′
3′ - CAAACCCATTATTTCCTTAA- 5′
5′- CATGGCCAAAGTACAAAGAGCGC- 3′
3′ - CGGTTTCATGTTTCT- 5′.

To generate a C-terminal deletion mutant of E4TF1-60, pET60-C305, the *Hae*II−*Bam*HI fragment of pET60 was excised and the remaining plasmid DNA was recircularized using the *Hae*II−*Bam*HI adaptor,

5′- CGAGGATTTCAGGATAG- 3′
3′- CGCGGCTCCTAAAGTCCTATCCTAG- 5′.

To construct pET60-C407, the DNA fragments encoding the sequence between amino acid residues 334 and 407 were amplified by PCR using two primers, 5′-GGACGCTCGAGACTGCATTT-3′ and 5′-GCACTG-GATCCTCAAAGAGTCTT-3′, and digested with both *Xho*I and *Bam*HI. The digested DNA fragment and the *Nco*I−*Xho*I fragment of pET60 were inserted into the *Nco*I−*Bam*HI sites of pET3d.

To generate plasmids pET53-C332 and pET53-C248 expressing C-terminal mutant proteins of E4TF1-53, 53-C332 and 53-C248 (see Figure 1), pET53 DNA was partially digested with *Pst*I and completely digested with *Bam*HI. The corresponding fragments between one of the *Pst*I sites and the *Bam*HI site were ligated to the *Pst*I−*Bam*HI adaptor,

5′- GAATCTGTGGATGGTTAG- 3′.
5′- ACGTCTTAGACACCTACCAATCCTAG- 3′

To prepare the E4TF1-53 deletion mutants, pET53-N133, pET53-N236, pET53-C135 and pET53-C153 were constructed. For pET52-N133 and pET53-N236, the appropriate DNA fragments were amplified by PCR using two primers, 5′-GCTGATGTACCCATGGAAAGTAA-3′ and 5′-ATTTGGATCCAACAGCTTCTTTATTAGTCT-3′, and 5′-GTGGCC-ATGGAAGAAGTAGTTACT-3′ and 5′-ATTTGGATCCAACAGCTT-CTTTATTAGTCT-3′, respectively. The amplified DNA fragments were digested with *Nco*I and *Bam*HI, and inserted into the *Nco*I−*Bam*HI sites of pET3d. To generate pET53-C135 and pET53-C153, the appropriate fragments were amplified by PCR using two primers, 5′-GCAAATGGA-GCTCCCTTTACT-3′ and 5′-TTGAGGATCCGAATTCAGTACGA-CCTTCGAT-3′, and 5′-GCAAATGGAGCTCCCTTTACT-3′ and 5′-ATTTGGATCCGCTAAGGAATTCGACGACCTTCGATTAAATC-TTCATTTCCATT-3′, respectively. The DNA fragments were digested with *Sac*I and *Bam*HI and inserted into the *Sac*I−*Bam*HI sites of pET53, which contained a unique *Sac*I site in the coding region of the E4TF1-53 N-terminus. The sequences of the PCR products were analyzed by DNA sequencing as described (Sanger *et al.*, 1977).

To construct pET53-I243/330, pET53 was digested with *Pst*I and the *Pst*I−*Pst*I fragment of the open reading frame was removed and recircularized. As for 53-I153/267, the DNA fragment encoding the E4TF1-53 C-terminal sequence was produced by digestion with *Eco*RI and *Bam*HI and inserted into the *Eco*RI−*Bam*HI sites of pET53-C153.

The plasmid that expresses E4TF1-F60/53, a chimeric protein consisting

of the ETS-DNA binding domain of E4TF1-60 and the C-terminal region of E4TF1-53, was constructed as follows. The DNA fragment encoding the C-terminal region specific for E4TF1-53 was amplified by PCR using two primers, 5′-ATTTGGATCCAACAGCTTCTTTATTAGTCT-3′ and 5′-TGCTGCAGGATCCGAGAGAGAAGCTCTTCAGAAACAG-3′. The product was digested with *Bam*HI, purified using agarose gel electrophoresis, and inserted into the *Bam*HI site of pET60-N293.

Expression and purification of E4TF1 subunit polypeptides

E4TF1-60, E4TF1-53 and the mutant proteins were expressed and purified as described previously (Watanabe *et al.*, 1993). We usually obtained 200 μl of extracts from 5 ml of cultured *E.coli*, 10 μl of which were resolved by SDS−PAGE. The fractionated proteins were eluted from the gel, denatured and renatured as described (Hager and Burgess, 1980) with a minor modification. Denatured proteins were renatured by dialysis against 50 mM Tris−HCl pH 7.9, containing 20% glycerol, 50 mM KCl, 1 mM EDTA, 1 mM EDTA and 1 mM DTT. After dialysis, the concentration of each protein was roughly adjusted to 50 μg/ml.

Gel shift assay

Gel shift assays were performed as described (Watanabe *et al.*, 1988) except that gel electrophoresis proceeded at 4°C. The DNA probe for this assay was constructed as follows: complementary oligonucleotides with a single E4TF1 recognition site were synthesized. The sequences were 5′-TTGTGGGAAAACGGAAGTGACGATTAA-3′ and 5′-TTAATCGTC-ACTTCCGTTTTCCCCACAA-3′. They were annealed, phosphorylated and cloned into the *Sma*I site of pUC19. To prepare the labeled probe, the plasmid DNA was digested with *Eco*RI and *Hin*dIII, then end-labeled with Klenow fragment and [α-^{32}P]dATP. The DNA fragment was purified by agarose gel electrophoresis. About 2 ng of the DNA probe was used for binding reactions.

In vitro transcription

E4TF1-depleted HeLa nuclear extracts were prepared using DNA affinity latex particles as described by Inomata *et al.* (1992). *In vitro* transcription assays were performed as described (Watanabe *et al.*, 1990). The reaction contained two DNA templates, 62.5 ng of pTF1-4(C_2AT) and 31.3 ng of pdML(C_2AT) (Watanabe *et al.*, 1990).

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