TFE3: A helix–loop–helix protein that activates transcription through the immunoglobulin enhancer μE3 motif

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The μE3 motif within the immunoglobulin heavy-chain enhancer is required for full enhancer activity and is known to bind one, or perhaps a family, of related ubiquitous nuclear proteins. Here, we present the isolation of a cDNA that encodes an apparently novel μE3-binding protein designated TFE3. The major open reading frame of the cDNA predicts a protein of 59 kD, with a leucine zipper situated adjacent to an *myc*-related motif that has been proposed to assume a helix–loop–helix structure. Both of these motifs have been shown (for other proteins) to facilitate protein-protein interactions and DNA binding. Expression of the cDNA in 3T3 cells stimulates transcription from an artificial promoter consisting of four μE3 sites linked to a TATA box and also augments transcription of a reporter gene when it is linked to multiple copies of a particular heavy-chain enhancer subfragment but not when it is linked to the intact enhancer. Using GAL4 fusion proteins, we mapped a strong transcription activation domain within TFE3 that is distinct from the leucine zipper and helix–loop-helix motifs and includes a potential negative amphipathic helix. Like the other μE3-binding proteins detected in nuclear extracts, in vitro-synthesized TFE3 also binds to the USF/MLTF site found in the adenovirus major late promoter.

The immunoglobulin heavy-chain (IgH) enhancer is thought to be crucial for the transcriptional activation of rearranged heavy-chain genes (Banerji et al. 1983; Gillies et al. 1983; Neuberger 1983; for review, see Atchison 1988). In transfection experiments, the IgH enhancer is capable of stimulating transcription from a variety of promoters, but only in cells of the B lineage (the IgH enhancer is also active in several T-cell lines). This activity has been shown to correlate well with the presence of approximately six protein binding sites within the enhancer. Four of these, μE1, μE2, μE3 and μE4, were defined initially by in vivo footprinting (Ephrussi et al. 1985; for review, see Calame and Eaton 1988). Two others, octamer (referring to the conserved octanucleotide ATGCAAAT; Singh et al. 1986; Staudt et al. 1986) and μEBP-E (Peterson et al. 1988), were defined first by in vitro binding assays. Deletions or mutations of these sites generally reduce overall enhancer activity (Lenardo et al. 1987; Kiledjian et al. 1988; Perez-Mutul et al. 1988; Tsao et al. 1988). Even though activity of the enhancer is restricted to B cells, only one of these sites (octamer) is known to bind B-cell-specific proteins (OTF-2/oct-2, OTF-2b; Landolfi et al. 1986; Staudt et al. 1986; Gerster

et al. 1987; Scheidereit et al. 1987; Schreiber et al. 1988) in addition to a ubiquitous protein (OTF-1/oct-1; Fletcher et al. 1987; Sturm et al. 1988). The other sites have been shown to bind only ubiquitous proteins. Specific binding to the μE4 motif has not been detected in vitro. The octamer element cannot account entirely for the B-cell specificity of the enhancer, however, because mutating or deleting it still results in a strong B-cell-specific enhancer (Lenardo et al. 1987; Kiledjian et al. 1988). Furthermore, recent evidence suggests that many different tissues, in addition to B cells, express one or more members of a large family of octa-binding proteins (Scholer et al. 1989a,b). Some reports suggest that the large octa-independent component of IgH enhancer activity is negatively regulated in non-B cells (Wasylyk and Wasylyk 1986; Kadesch et al. 1986; Imler et al. 1987; Weinberger et al. 1988).

One of the enhancer elements bound by a ubiquitous DNA-binding activity is the μE3 site. Studies of mutant enhancers have indicated that this site alone may be responsible for as much as 30% of the enhancer's activity in B cells (Lenardo et al. 1987; Kiledjian et al. 1988; Perez-Mutul et al. 1988; Tsao et al. 1988). The site was also shown to contribute to the low level of enhancer activity observed in non-B cells (Kiledjian et al. 1988). The corresponding DNA-binding activity, NF-μE3, was

thought initially to represent a single protein (Sen and Baltimore 1986); however, purification has since revealed perhaps as many as three related proteins of similar size (42.5–45 kD) that interact with the motif in various oligomeric forms (Peterson and Calame 1989), and a related activity has even been described in yeast (Beckmann and Kadesch 1989). For simplicity, we will use the term NF-µE3 to describe the mammalian µE3-binding activity derived from nuclear extracts, which may represent multiple proteins. We have noticed that a DNA sequence that bears remarkable similarity to the µE3 motif is also found in the adenovirus major late promoter. This site in the major late promoter is bound by the transcription factor USF (or MLTF), a 43- to 46-kD protein (Chodosh et al. 1986; Sawadogo et al. 1988). In fact, the USF/MLTF-binding site is more related to the µE3 site (11 of 12 nucleotides) than the κE3 site within the κ light-chain enhancer (7 of 7 nucleotides), which is known to bind NF-µE3 (Sen and Baltimore 1986). This suggests the interesting prospect that USF/MLTF and NF-µE3 activities are related or, perhaps, are one in the same.

As a first step toward developing a general understanding of how cell-type specificity is imposed on apparently ubiquitous DNA-binding proteins and, specifically for the case of the µE3 site, to elucidate the relationship between NF-µE3 and USF/MLTF, we isolated a cDNA that encodes a µE3-binding protein. The encoded protein is almost certainly not USF/MLTF or NF-µE3, although it does bind to a USF/MLTF-binding site. Designated transcription factor E3 (TFE3), the encoded protein represents a novel DNA-binding protein that functions as a positive-acting transcription factor. Furthermore, it possesses two structural motifs that may mediate interactions with other proteins.

Results

Isolation of a cDNA encoding a µE3-binding protein

To identify cDNAs that encode µE3-binding proteins, we screened a human B-cell-derived λgt11 cDNA library with labeled oligonucleotides carrying the µE3-binding site (see Methods). We isolated one phage, designated λ3, that expressed a β-galactosidase fusion protein with the ability to bind specifically to the µE3-containing oligonucleotide. As shown in Figure 1A, mobility-shift assays using λ3 lysogen extracts detected a slow migrating activity that bound the oligonucleotide carrying the wild-type µE3 sequence (WT, lane 5), but not a similar oligonucleotide carrying a 3-base change mutant µE3 site (MUT, lane 6). This specificity was mimicked by two shifted bands found with nuclear extracts of HeLa cells (lanes 7 and 8), and no complex with this specificity was observed with λgt11 lysogen extracts (lanes 3 and 4). Western and Southwestern analyses of lysogen extracts resolved by SDS-polyacrylamide gel electrophoresis confirmed that the µE3-binding activity comigrated with a high-molecular-weight β-galactosidase fusion protein (data not shown).

To explore further the nature of the binding to the µE3 site, we carried out a dimethylsulfate (DMS) interference analysis. Because of the relatively poor binding obtained with the bacterial lysogen extracts, and for convenience, we employed a different fusion protein that was synthesized in vitro. In this case, a segment of the λ3 cDNA insert was removed from λgt11 and fused to the first 147 amino acids of the yeast GAL4 protein (GAL4λ3-A) in a vector carrying the promoter for SP6 RNA polymerase adjacent to the GAL4 sequences. When this vector was transcribed by SP6 RNA polymerase, and the synthesized RNA was subsequently translated in a reticulocyte lysate, a suitably stable complex could be obtained with a fragment derived from the IgH enhancer (for a representative mobility-shift assay using this protein, see Methods and Fig. 6, lane 17, below). As shown in Figure 1B, the GAL4λ3-A fusion protein gave a DMS interference pattern coincident with the µE3 site that was nearly identical to that described previously for NF-µE3 activity in nuclear extracts (Sen and Baltimore 1986; Peterson and Calame 1987).

λ3 cDNA encodes a 59-kD protein with a leucine zipper adjacent to a putative helix–loop–helix motif related to c-myc

The sequence of the 2.5-kb λ3 cDNA is shown in Figure 2. It contains one major open reading frame capable of encoding a protein of 59 kD. Southern hybridization confirmed that the cDNA is encoded by the human genome. Northern analysis identified an RNA of ~2.5 kb that, in addition to human B cells, is present in a wide variety of cell types (e.g., HeLa cells) and mouse tissues (e.g., liver and kidney; data not shown). Primer extension experiments indicated that the λ3 cDNA terminates ~150 nucleotides from the true 5' end (data not shown). Hence, at present, we cannot say whether the first encoded methionine (position 41) represents the normal site of translation initiation or is internal to the protein. If it is the initiating methionine, the protein would have a calculated molecular weight of ~55 kD. Even though the flanking nucleotides of this translation initiation site are not optimal (Kozak 1984), as will be shown below, this AUG does have the capacity to initiate the translation of functional protein in vivo. Examination of the 3' end of the cDNA failed to reveal an apparent polyadenylation signal or poly(A) tail.

A comparison of the amino acid sequence to those of other DNA-binding proteins and transcription factors revealed three interesting motifs. The first, encompassing amino acids 134–199, is a region that bears a high degree of identity to the myc family of proteins. Initially described in MyoD by Tapscott et al. (1988) as a myc similarity region (MSR), it has been proposed by Baltimore and co-workers (Murre et al. 1989a,b) that certain classes of MSRs define amphipathic helix–loop–helix structures that mediate protein oligomerization and DNA binding. The helix–loop–helix structure has been found in several other transcription factors, including MyoD, myogenin, three immunoglobulin en-
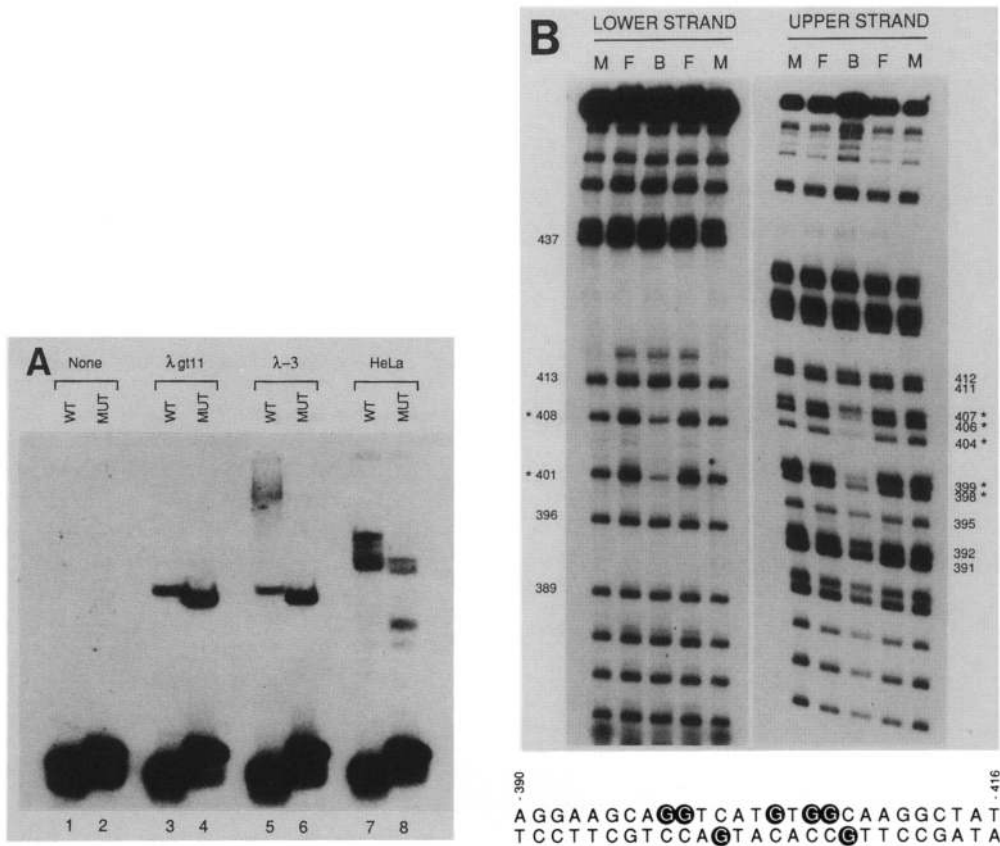
Figure 1. The λ3 fusion proteins bind to the µE3 site of the IgH enhancer. (*A*) Mobility-shift assays using bacterial lysogen extracts and HeLa cell nuclear extracts. Labeled oligonucleotides containing a wild-type (wt) or a mutant (mut) µE3 site were incubated with either no extract (lanes *1* and *2*), λgt11 lysogen extracts (lanes *3* and *4*), λ3 lysogen extracts (lanes *5* and *6*), or HeLa nuclear extracts (lanes *7* and *8*). (*B*) Methylation interference assays employed in vitro-synthesized GAL4λ3-A fusion protein and IgH enhancer fragment 20, as described in Methods. A representative mobility-shift assay with this protein is shown in Fig. 6 (lane *17*) below. The free (F) and the bound (B) forms of the lower and upper strand of the a fragment are indicated. Markers were derived from a fragment subjected to a Maxam–Gilbert sequencing reaction. Cleaved guanosine residues are indicated by the numbering system of Gillies et al. (1983). (*) Guanosine residues that diminish binding when methylated. A summary of the results is presented below the autoradiograph. Points of interference are indicated by circled G residues, which are identical to those described for partially purified murine NF-µE3 (Peterson and Calame 1987) and, except for guanosine 398, identical to those described in vivo (Ephrussi et al. 1985) and for NF-µE3 in crude nuclear extracts (Sen and Baltimore 1986).

hancer µE5/κE2-binding proteins, a recently described gene (*lyl-1*) adjacent to a chromosome translocation in T-cell acute lymphoblastic leukemia, and several *Drosophila* genes associated with neuronal development (Tapscott et al. 1988; Mellentin et al. 1989; Murre et al. 1989a; Henthorn et al. 1990). A comparison of *myc*-related helix–loop–helix sequences of the mammalian proteins (Fig. 3) reveals a remarkable degree of identity and similarity. The second motif predicted by the λ3 cDNA sequence is a leucine zipper (Landschulz et al. 1988), and it is situated immediately adjacent to the MSR. It consists of four leucines spaced at 7-amino-acid intervals, beginning at amino acid 202. Leucine zippers have been found in a number of eukaryotic transcription factors and also have been shown to facilitate protein–protein interactions (e.g., Kouzarides and Ziff 1988, 1989; Gentz et al. 1989; Landschulz et al. 1989). It has been suggested that leucine zippers mediate coiled-coil interactions and can represent a subset of the three-four

rule, where every third and fourth amino acid, defining a broad face of an α-helix, is hydrophobic (O'Shea et al. 1989). Consistent with a possible role in mediating such a coiled-coil interaction, the three–four rule is adhered to beginning in the middle and extending slightly beyond the λ3 zipper region. The third motif found at the carboxyl end of the λ3 protein (amino acids 368–536) is a motif defined merely by its being rich in proline residues (16%) and arginine residues (14%). Although such a region may, by definition, be devoid of structure, a proline-rich region has been shown to define a transcription activation domain in the CCAAT-binding factor CTF (Mermod et al. 1989).

The λ3 protein activates transcription through artificial and natural µE3-binding sites

To test directly whether the λ3 protein encodes a transcription factor, we expressed the cDNA in mammalian

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     GAATTCGGGCGGGCTTCCCAGGCCCTCACCCCACCGCCGGGGAAAGCAAGTGCCCAGCCACTGCCTGCCCCTGAGGCTGCCCACACTACCGGCCCCACAGGCAGTGCGCCCAACAGCCCC  120
   1 GluPheGlyArgAlaSerGlnAlaLeuThrProProProGlyLysAlaSerAlaGlnProLeuProAlaProGluAlaAlaHisThrThrGlyProThrGlySerAlaProAsnSerPro

     ATGGCGCTGCTCACCATCGGGTCCAGCTCAGAGAAGGAGATTGATGATGTCATTGATGAGATCATCAGCCTGGAGTCCAGTTACAATGATGAAATGCTCAGCTATCTGCCCGGAGGCACC  240
  41 MetAlaLeuLeuThrIleGlySerSerSerGluLysGluIleAspAspValIleAspGluIleIleSerLeuGluSerSerTyrAsnAspGluMetLeuSerTyrLeuProGlyGlyThr

     ACAGGACTGCAGCTCCCCAGCACGCTGCCTGTGTCAGGGAATCTGCTTGATGTGTACAGTAGTCAAGGCGTGGCCACACCAGCCATCACTGTCAGCAACTCCTGCCCAGCTGAGCTGCCC  360
  81 ThrGlyLeuGlnLeuProSerThrLeuProValSerGlyAsnLeuLeuAspValTyrSerSerGlnGlyValAlaThrProAlaIleThrValSerAsnSerCysProAlaGluLeuPro

     AACATCAAACGGGAGATCTCTGAGACCGAGGCAAAGGCCCTTTTGAAGGAACGGCAGAAGAAAGACAATCACAACCTAATTGAGCGTCGCAGGCGATTCAACATTAACGACAGGATCAAG  480
 121 AsnIleLysArgGluIleSerGluThrGluAlaLysAlaLeuLeuLysGluArgGlnLysLysAspAsnHisAsnLeuIleGluArgArgArgArgPheAsnIleAsnAspArgIleLys

     GAACTGGGCACTCTCATCCCTAAGTCCAGTGACCCGGAGATGCGCTGGAACAAGGGCACCATCCTGAAGGCCTCTGTGGATTATATCCGCAAGCTGCAGAAGGAGCAGCAGCGCTCCAAA  600
 161 GluLeuGlyThrLeuIleProLysSerSerAspProGluMetArgTrpAsnLysGlyThrIleLeuLysAlaSerValAspTyrIleArgLysLeuGlnLysGluGlnGlnArgSerLys

     GACCTGGAGAGCCGGCAGCGATCCCTGGAGCAGGCCAACCGCAGCCTGCAGCTCCGAATTCAGGAACTAGAACTGCAGGCCCAGATCCATGGCCTGCCAGTACCTGGGACTCCAGGGCTG  720
 201 AspLeuGluSerArgGlnArgSerLeuGluGlnAlaAsnArgSerLeuGlnLeuArgIleGlnGluLeuGluLeuGlnAlaGlnIleHisGlyLeuProValProGlyThrProGlyLeu

     CTTTCCTTGGCCACGACTTCAACTTCTGACAGCCTCAAGCCAGAGCAGCTGGACATTGAGGAGGAGGGCAGGCCAGGCGCACGAACGTTCCATGTAGGGGGGGGACCTGCCCAGAATGCT  840
 241 LeuSerLeuAlaThrThrSerThrSerAspSerLeuLysProGluGlnLeuAspIleGluGluGluGlyArgProGlyAlaArgThrPheHisValGlyGlyGlyProAlaGlnAsnAla

     CCCCATCAGCAGCCCCCTGCACCGCCCTCAGATGCCCTTCTGGACCTGCACTTTCCCAGCGACCACCTGGGGGACCTGGGAGACCCCTTCCACCTGGGGCTGGAGGACATTCTGATGGAG  960
 281 ProHisGlnGlnProAlaProProSerAspAlaLeuLeuAspLeuHisPheProSerAspHisLeuGlyAspLeuAspProPheHisLeuGlyLeuGluAspIleLeuMetGlu

     GAGGAGGAGGGGGTGGTGGGAGGACTGTCGGGGGGTGCCCTGTCCCCACTGCGGGCTGCCTCCGATCCCCTGCTCTCTTCAGTGTCCCTGCTGTCTCCAAGGCCAGCAGCCGCCGCAGCA  1080
 321 GluGluGluGlyValValGlyGlyLeuSerGlyGlyAlaLeuSerProLeuArgAlaAlaSerAspProLeuLeuSerValSerLeuLeuSerProArgProAlaAlaAlaAlaAla

     GCTTCAGCATGGAAGAGGAGTCCTGATCAGGCCTCACCCCTCCCCTGGACTTTCCCACCCAGGAAAGGAGGACCATGCAGGATGAGGCCCCGCCTTTTCCCCCACCCTCCCATGAGACTG  1200
 361 AlaSerAlaTrpLysArgSerProAspGlnAlaSerProLeuProTrpThrPheProProArgLysGlyGlyProCysArgMetArgProArgLeuPheProHisProProMetArgLeu

     CCCAGGTATCTGGGAAGAGGAGATGTGATCAGGCCCCACCCCTGTAATCAGGCAAGGAGGAGGAGTCAGATGAGGCCCTGCACCTTCCCCAAAGGAACCGCCCAGTGCAGGTATTTCAGA  1320
 401 ProArgTyrLeuGlyArgGlyAspValIleArgProHisProCysAsnGlnAlaArgArgArgSerGlnMetArgProCysThrPheProLysGlySerAlaGlnCysArgTyrPheArg

     AGGAGAAGGCTGGAGAAGGACATGAGATCAGGGCCTGCCCCCTGGGGATCACAGCCTCACCCCTGCCCCTGTGGGACTCATCCTTGCCCAGGTGAGGGAAGGAGACAGGATGAGGTCTCG  1440
 441 ArgArgArgLeuGluLysAspMetArgSerGlyProAlaProTrpGlySerGlnProHisProCysProCysGlyThrHisProCysProGlyGlnGlyArgArgGlnAspGluValSer

     ACCCTGTCCCCTAGGGACTGTCCTAGCCAGGTCTCCTGGGAAAGGGAGATGTCAGGATGTTGCTCCATCCTTTGTCTTGGAACCACCAGTCTAGTCCGTCCTGGCACAGAAGAGGAGTCA  1560
 481 ThrLeuSerProArgAspCysProSerGlnValSerTrpGluArgGluMetSerGlyCysCysSerIleLeuCysLeuGlyThrThrSerLeuValArgProGlyThrGluGluGluSer

     AGTAATGGAGGTCCCAGCCCTGGGGGTTTAAGCTCTGCCCCTTCCCCATGAACCCTGCCCTGCTCTGCCCAGGCAAGGAACAGAAGTGAGGATGAGACCCAGCCCCTTCCCCTGGGAACT  1680
 521 SerAsnGlyGlyProSerProGlyGlyLeuSerSerAlaProSerPro  536

     CTCCTGGCTTCTAGGAATGGAGGAGCCCAGGCCCCACCCCCTTCCCTATAGGAACAGCCCAGCACAGGTATTTCAGGTGTGAAAGAATCAGTAGGACCAGGCCACCGCTAAGTGCTTGTG  1800

     GAGATCACAGCCCCACCCTTGTCCCTCAGCAACATCCCATCTAAGCATTCCACACTGCAGGGAGGAGTGGTACTTAAGCTCCCCTGCCTTAACCTGGGACCAACCTGACCTAACCTAGGA  1920

     GGGCTCTGAGCCAACCTTGCTCTTGGGGAAGGGGACAGATTATGAAATTTCATGGATGAATTTTCCAGACCTATATCTGGAGTGAGAGGCCCCCCACCCCTTGGGCAGAGTCCTGCCTTC  2040

     TTCCTTGAGGGGCAGTTTGGGAAGGTGATGGGTATTAGTGGGGGACTGAGTTCAGGTTACCAGAACCAGTACCTCAGTATTCTTTTTCAACATGTAGGGCAAGAGGATGAAGGAAGGGGC  2160

     TATCCTGGACCTCCCCAGCCCCAGGAAAAACTGGAAGCCTTCCCCCAGCAAGGCAGAAGCTTGGAGGAGGGTTGTAAAAGCATATTGTACCCCCTCATTTGTTTATCTGATTTTTTTATTC  2280

     GTCCGCATACTGAGAATCTAGGCCACCCCAACCTCTGTTCCCCACCCAGTTCTTCATTTGGAGGAATCACCCCATTTCAGAGTTATCAAGAGACACTCCCCCCTCCATTCCCACCCCTCA  2400

     TACCTACACCCAAGGTTGTCAGCTTTGGATTGGTCGGGGCCAGGCCCCATGGAGGGTATACTGAGGGGTCTATAGGCCC  2479
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Figure 2. Nucleotide sequence of the λ3 cDNA and its deduced amino acid sequence. The first methionine in the sequence is amino acid 41. The *myc* similarity region (MSR or helix–loop–helix) spans residues 134–199, and the leucine zipper begins at amino acid 202. The proline/arginine-rich region begins at amino acid 368. The EMBL accession number for human TFE3 is 51330.

cells and measured the response of a reporter gene carrying μE3-binding sites. The plasmid pSV2A-λ3 (Fig. 4A) contains the λ3 cDNA under control of the SV40 early promoter. The cDNA was inserted in such a way that its expression would rely on translation from its own AUG (amino acid 41 in the open reading frame). Because the cDNA lacked introns and an apparent polyadenylation signal, the SV40 small-t antigen intron and early polyadenylation signals were provided 3' to the cDNA. Initially, we employed three reporter plasmids. The first contained the TATA box from the liver/bone/kidney alkaline phosphatase gene promoter linked to the bacterial chloramphenicol acetyltranferase (CAT) gene (Weiss et al. 1988). The second linked four tandem copies of the wild-type μE3 oligonucleotide to the TATA box. The third linked four tandem copies of the mutant μE3 oligonucleotide to the TATA box. As shown in Figure 4A, cotransfection of mouse 3T3 cells with increasing amounts of pSV2A-λ3, along with the reporter plasmid carrying the normal μE3 oligonucleotides, resulted in increasing levels of CAT activity (lanes 4–6). No activation was observed with the TATA box alone (lanes 1–3) or when the TATA box was linked to oligonucleotides carrying the mutant μE3 sites (lanes 7–9). The maximum level of stimulation in this experiment gave a CAT activity similar to that obtained with the SV40 early promoter, in which expression of CAT (lane 10) is increased. Low levels of activity are obtained with the normal oligonucleotides in the absence of cotransfected pSV2A-λ3 plasmid (cf. lanes 4 and 7). This is attributable to endogenous μE3 transcription factors, possibly related to the λ3 protein (D. Ruezinsky and T. Kadesch, unpubl.).

These results demonstrate clearly that the λ3-encoded protein can activate transcription through its cognate binding site; therefore we will refer to this protein as transcription factor E3 (TFE3).

The former experiments leave unanswered the question of whether TFE3 can activate transcription via the IgH enhancer. To address this, we employed three additional reporter plasmids in our transfection experiments. The first contained the human β-globin promoter linked to the CAT gene (plasmid βG-CAT). The second carried 12 tandem copies of a wild-type IgH enhancer (fragment 2) positioned 3' to the βG-CAT transcription unit [plasmid βG-CAT-(2)$_{12}$]. The third carried 12 tandem copies of a subfragment of the enhancer (fragment 12) linked to the βG-CAT transcription unit (plasmid βG-

CAT(12)$_{12}$]. Fragment 12 carries only the µE3-binding site and the enhancer 'core' sequences (Kadesch et al. 1986). The activity of fragment 12 (and that of related fragments) in non-B cells led to the hypothesis that it was devoid of negative motifs that repressed enhancer activity in non-B cells (Kadesch et al. 1986; Wasylyk and Wasylyk 1986; Weinberger et al. 1988). It has been shown previously that multiple copies of the enhancer can lead to detectable enhancer activity in non-B cells (Kiledjian et al. 1988). Hence, we reasoned that for transfection of 3T3 cells, these plasmids bearing multiple enhancers may be more sensitive to *trans*-activation.

As shown in Figure 4B, cotransfection of 3T3 cells with increasing amounts of pSV2A-λ3 and the reporter plasmid carrying fragment 12 led to increased expression from the βG-CAT transcription unit (lanes 7–9). No activation was observed with reporter plasmids lacking the enhancer (lanes 4–6) or with plasmids carrying the wild-type enhancer (lanes 10–12). Note that in the absence of cotransfected pSV2A-λ3, enhancer fragment 12 is more active than enhancer fragment 2 (cf. lanes 7 and 10). Even though the level of TFE3-mediated activation of enhancer fragment 12 was lower than that obtained with the [E3]$_4$–TATA promoter (lanes 1–3), the effect was reproducible. We were unable to obtain similar results with cotransfections into the B cell line P3-X63Ag8 (data not shown). However, in those experiments, the overall *trans*-activation potential of even the [E3]$_4$-TATA promoter was reduced; thus, it is likely that this negative result represented a technical limitation. Hence, we conclude that TFE3 can activate transcription through the µE3 site in the IgH enhancer in 3T3 cells but cannot do so if the intact enhancer is used. This result is consistent with models invoking negative regulation of the enhancer.

Under certain conditions it may be useful to synthesize very high levels of transcription factors. We explored this possibility for TFE3 by creating a plasmid in which TFE3 would autoregulate its own synthesis through a positive-feedback loop. The plasmid pFBE3 contains the λ3 cDNA inserted downstream of the [E3]$_4$–TATA promoter. We determined the ability of pFBE3 to activate expression of the [E3]$_4$–TATA promoter linked to the CAT gene, and compared this to the activating ability of pSV2A-λ3. In the absence of autoregulation, pFBE3 would be expected to express very low levels of TFE3 compared to pSV2A-λ3 (see Fig. 4A, lanes 4 and 10). However, as shown in Figure 4C, pFBE3 functions as a better activator than pSV2A-λ3 (cf. lanes 4 and 5 or lanes 6 and 7). Hence, in cells producing artificially high levels of TFE3, the [E3]$_4$–TATA promoter becomes more efficient than the SV40 early promoter.

TFE3 has a strong transcription activation domain in a region that contains a potential negative amphipathic helix

We sought to map the region(s) of TFE3 directly responsible for its ability to activate transcription. To do this we fused either the full-length λ3 protein or subregions
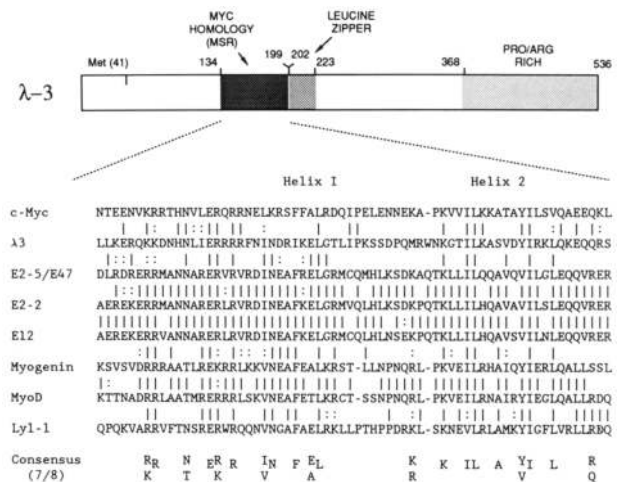

Figure 3. Sequences of MSR (or helix–loop–helix) of various mammalian proteins. (*Top*) Schematic representation of the λ3 protein. The relevant regions of the following proteins are shown. Human c-*myc* (amino acids 341–403; Battey et al. 1983), myogenin (amino acids 76–137; Wright et al. 1989), MyoD (amino acids 104–167; Davis et al. 1987), *lyl-1* (amino acids 132–196; Mellentin et al. 1989), and the µE5/κE2 enhancer-binding proteins E47 and E12 (amino acids 331–395; Murre et al. 1989a), E2-5 (amino acids 474-539; Henthorn et al. 1990) and E2-2 (amino acids 514–579; Henthorn et al. 1990). Identical amino acids are indicated by vertical lines, and conserved amino acids are represented by double dots. The proposed amphipathic helices I and II (Murre et al. 1989a) are indicated. The consensus sequence represents amino acids which are found in seven of the eight possible positions.

to the DNA-binding domain of the yeast GAL4 protein (GAL4 amino acids 1–147; Keegan et al. 1986). Because the truncated GAL4 protein alone cannot activate transcription, the presence of an activation domain is indicated by the ability of the GAL4 fusion protein to mediate transcription activation of a reporter gene through GAL4-binding sites (Keegan et al. 1986; Ma and Ptashne 1987). We obtained three reporter genes for these studies (Lillie and Green 1989). One contains the adenovirus E1B TATA box linked to the CAT gene; the other two contain either one or five copies of a GAL4-binding site linked to the E1B TATA box.

The various GAL4 fusion proteins employed and the results of two representative experiments are summarized in Figure 5A. When the entire open reading frame of the λ3 cDNA was fused to the GAL4 DNA-binding domain (GAL4λ3) and cotransfected into 3T3 cells with the different reporter plasmids, transcription was stimulated 50- to 100-fold when the reporter plasmids carried GAL4-binding sites. Good activation was seen with a single GAL4-binding site, and this level was not increased when five GAL4-binding sites were used instead of one. This restriction may be attributable to stearic hindrance. No activation of any of the reporter plasmids was seen with the GAL4-binding domain alone

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Figure 4. The λ3 protein activates transcription through a μE3 site in vivo. (*A*) Activation of a promoter containing four copies of the μE3 site. A schematic diagram of the pSV2A-λ3 expression plasmid is shown. The λ3 sequences that presumably encode protein upon transfection are shown (as in Fig. 3) as wide rectangles (translation initiation begins at Met-41). The noncoding segments of the cDNA are shown as thin rectangles. t and A_n represent the positions of the SV40 small-t antigen intron and early polyadenylation signal, respectively. Reporter plasmids (1 μg) carrying either a TATA box linked to the bacterial CAT gene (TATA; lanes *1–3*), four tandem copies of the wild-type μE3 oligonucleotide linked to TATA–CAT ([E3]₄–TATA; lanes *4–6*), or four copies of the mutant μE3 oligonucleotide ([mut-E3]₄–TATA; lanes *7–9*) were transfected into 3T3 cells along with the indicated amounts of pSV2A-λ3 plus 5 μg pCH110 and pUC DNA to bring the total amount of transfected DNA to 20 μg. Cells were harvested and assayed for CATase activity 2 days after transfection. Lane *10* shows the CATase activity obtained from cells transfected with 1 μg pSV2Acat, which expresses the CAT gene under control of the SV40 early promoter. (*B*) pSV2A-λ3 stimulates transcription through an IgH enhancer subfragment. Reporter plasmids (1 μg) containing the TATA box linked to four μE3 sites ([E3]₄–TATA-CAT; lanes *1–3*) or carrying the CAT gene linked to the human β-globin promoter either alone (βG–CAT; lanes *4–6*), linked to 12 copies of the IgH enhancer fragment 12 positioned 3′ [βG–CAT–(12)₁₂; lanes *7–9*], or linked to 12 copies of enhancer fragment 2 positioned 3′ [βG-CAT–(2)₁₂; lanes *10–12*] were transfected into 3T3 cells, along with the indicated amounts of pSV2A-λ3, and assayed for CATase as described above. (*C*) Overexpression of the λ3 protein through a positive feedback loop. Reporter plasmids (0.5 μg) were transfected into 3T3 cells alone (lane *1*), or along with the indicated amounts of pSV2A-λ3 (lanes *2, 4,* and *6*) or the positive feedback plasmid pFBE3 in which the λ3 cDNA is expressed from a [E3]₄–TATA promoter (lanes *3, 5–7*), and assayed for CATase, as described above.
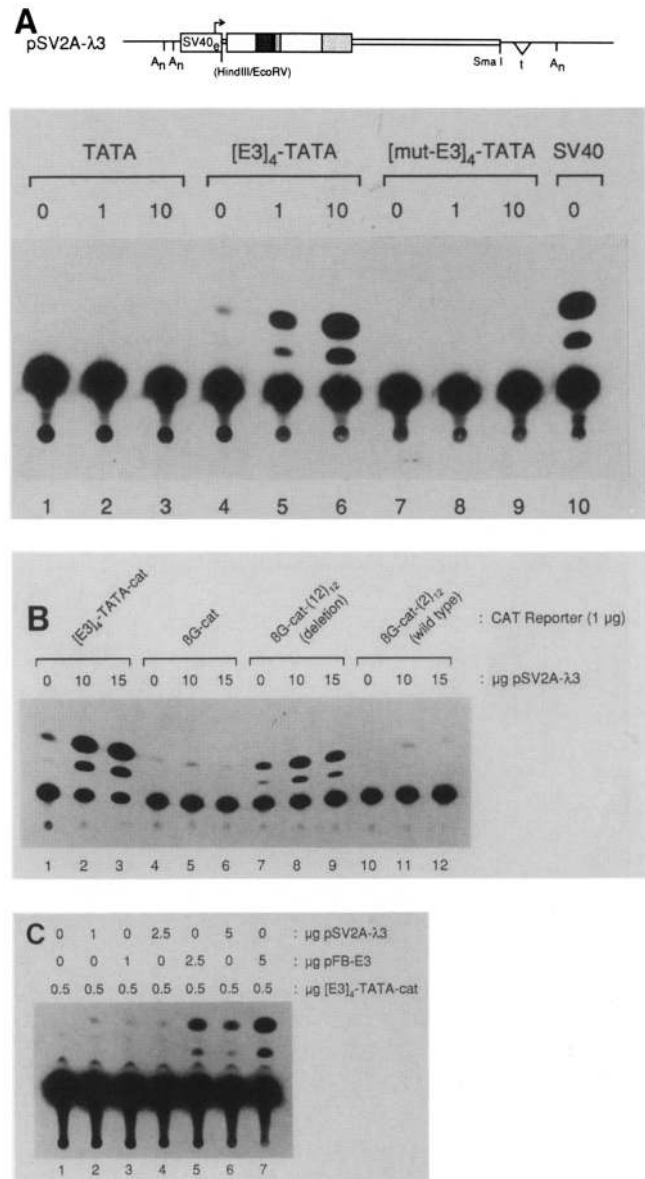
(GAL4₁₋₁₄₇). The maximum level of stimulation with intact TFE3 was roughly the same as that obtained for a fusion protein of GAL4 and an activating region of the adenovirus E1A protein (GAL4–E1A, E1A amino acids 121–223; Lillie and Green 1989). If the GAL4 DNA-binding domain was fused in-frame to TFE3 at a position initiating at amino acid 126, including the entire MSR and leucine zipper (GAL4λ3-Δ1), only moderate levels of activation were observed (~10-fold lower than those found with the intact protein), which did, however, increase as the number of GAL4-binding sites was increased from one to five. In contrast, when the amino-terminal 126 amino acids of the protein were tested (GAL4λ3-Δ2), extremely high levels of activation resulted (>1000-fold). In this case, there was a striking increase in activity when the number of GAL4-binding sites on the reporter plasmid was increased from one to

five. Perhaps this dramatic increase reflects cooperative binding of these particular fusion proteins.

The results with the GAL4–TFE3 fusion proteins indicate the presence of a very strong activation domain within the first 126 amino acids of the λ3 open reading frame. However, because the TFE3 protein expressed in our pSV2Aλ3 transfections is also active and begins with the methionine at position 41, we suspect that the activation domain actually falls between amino acids 41 and 126. When we examined this sequence closely, we noted a region with probable α-helical character (Chou and Fassman 1974) with an overall net negative charge of -7. In fact, as diagrammed in Figure 5B, it contains a small polypeptide (12 amino acids) that is likely to form a good amphipathic helix with one negative surface and one hydrophobic surface. These types of negative amphipathic helices are thought to define an important
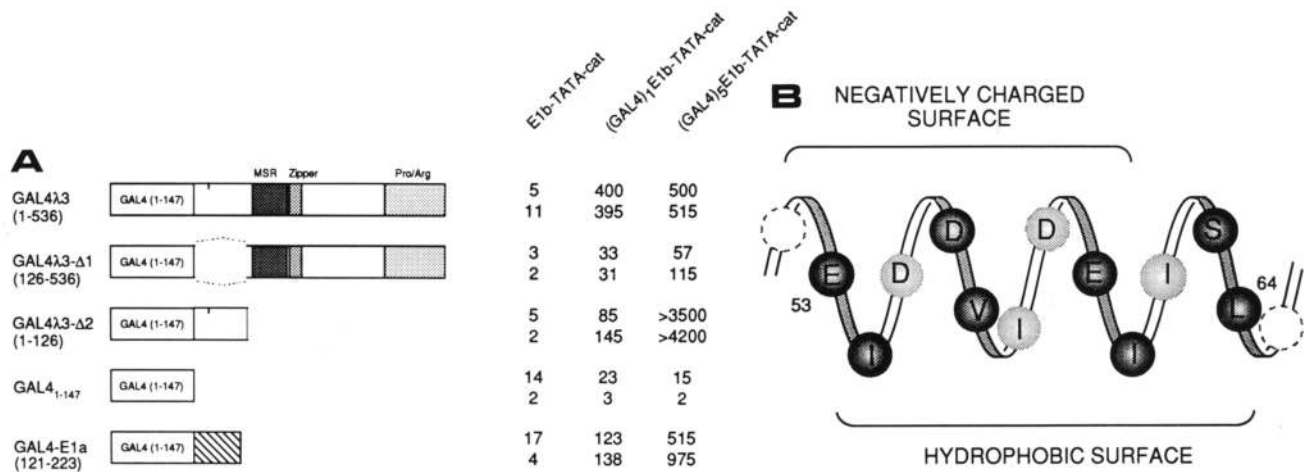
Figure 5. TFE3 contains a strong transcription activation domain. (*A*) All GAL4—TFE3 fusion plasmids are diagrammed schematically with the encoded amino acids indicated. Plasmid GAL4-E1A bears an activation domain of the adenovirus E1A protein. The reporter plasmids (Lillie and Green 1989) contained the CAT gene linked to an adenovirus E1B promoter TATA box which, in turn, is linked to either no GAL4-binding sites (E1B–TATA–cat), one GAL4-binding site [(GAL4)₁E1B–TATA–cat], or five GAL4-binding sites [(GAL4)₅E1B–TATA–cat]. Transfections into 3T3 cells were carried out using 10 µg of each plasmid plus 5 µg pCH110. CATase values given are from two separate experiments and expressed in arbitrary units relative to β-galactosidase activities. (*B*) Schematic representation of the putative negative amphipathic helix.

class of eukaryotic transcription activation domains (Giniger and Ptashne 1987; Ptashne 1988).

TFE3 is distinct from NF-µE3, yet both DNA-binding activities recognize a USF/MLTF motif

To examine the relationship between TFE3 and NF-µE3, we carried out a comparison of the two DNA-binding activities. As a source of TFE3 we employed a full-length protein, βG-λ3, synthesized in vitro. The in vitro-synthesized βG-λ3 protein contains all of the amino acids specified by the λ3 open reading frame plus an amino-terminal methionine derived from a β-globin gene segment and an isoleucine encoded by a polylinker fragment (see Methods). As a source of NF-µE3-binding activity, we used nuclear extracts prepared from the human B-cell line Namalwa. We carried out mobility-shift assays employing a labeled fragment of the IgH enhancer and a variety of competitor oligonucleotide DNAs. The results are shown in Figure 6. In Namalwa extracts we observed a slow migrating doublet that was sensitive to an oligonucleotide competitor carrying the µE3 site (lanes 1 and 2). Competition was not observed with an oligonucleotide carrying a mutant µE3 site (lane 3), and identical complexes were observed with a fragment carrying the κE3 site (not shown). Hence, we conclude that this doublet represents NF-µE3 activity (Sen and Baltimore 1986). This activity was also sensitive to competition by two different oligonucleotides that bear USF/MLTF-binding sites. The first carries the DNA sequence from the adenovirus major late promoter (USF, lane 5). The second contains a µE3 oligonucleotide that was changed at a single base pair to specify a USF/MLTF site (µE3→USF, lane 6). These results confirm that NF-µE3 has a binding specificity similar to that of USF/

MLTF. In vitro-synthesized βG-λ3 showed the same binding specificity as NF-µE3 (lanes 10–14), including the ability to bind to a κE3 site (data not shown).

Complexes formed with βG-TFE3 have a slower mobility than those formed with NF-µE3 (cf. Fig. 6, lanes 8 and 10). This is not surprising considering that the βG-TFE3 protein is calculated to be at least 13 kD larger than NF-µE3 (59 versus 42.5–46 kD). The predicted size of the βG-TFE3 protein is consistent with its mobility on SDS-polyacrylamide gels (data not shown). Even if we assume that the βG-TFE3 protein represents an artificially large version of TFE3 (i.e., that Met-41 represents the first codon of the normal protein), we would calculate the size of TFE3 to be ~55 kD, which is still much larger than NF-µE3.

Although NF-µE3 is the only µE3-binding activity detected in mobility-shift assays with crude nuclear extracts, data that support the existence of an additional, larger µE3-binding protein are shown in Figure 7. HeLa nuclear extracts were resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose and exposed to either the wild-type µE3 oligonucleotide [wt oligo (Fig. 7A)] or the mutant µE3 oligonucleotide [mut. oligo (Fig. 7B)]. The filters were subjected to a denaturation/renaturation cycle and probed using exactly the same procedure we used to screen the λgt11 cDNA library for TFE3. This experiment identified a 62- to 68-kD protein (bold arrow), and perhaps a slightly larger one (small arrow), that binds specifically to the oligonucleotide carrying the normal µE3 site. Even though the major binding protein is larger than what is known of TFE3, if we consider the resistance of each binding activity to denaturation, that the λ3 cDNA is not quite full length, and the potential for post-translational modifications, we feel that it probably does represent TFE3.
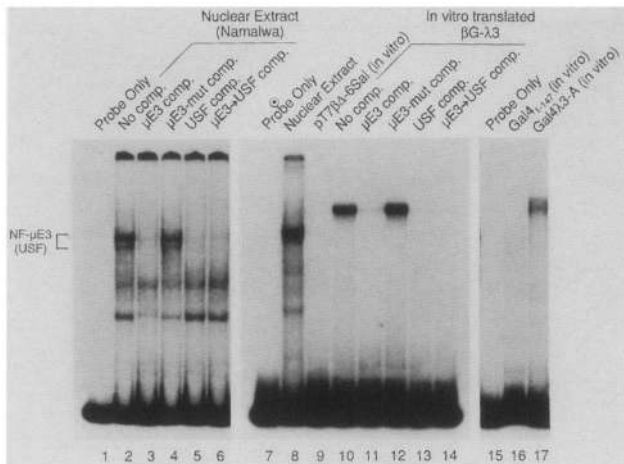
Figure 6. NF-μE3 and TFE3 synthesized in vitro bind to the USF/MLTF site of the adenovirus major late promoter. Mobility-shift assays used IgH enhancer fragment 12 as probe and various unlabeled oligonucleotide DNAs as competitors. Assays employed Namalwa nuclear extracts or in vitro-synthesized TFE3 proteins, as indicated (see Methods). (Lanes 1, 7, and 15) Probe alone; (lanes 2 and 8) probe plus Namalwa nuclear extract without competitor; (lanes 10, 16, and 17) probe plus in vitro-synthesized proteins expressed from TFE3-containing plasmids without competitor; (lane 9) probe plus in vitro-generated protein from the parental plasmid lacking the L3-cDNA (pT7βΔ-6Sal) without competitor. All other lanes contained probe plus the indicated sources of protein and competitor oligonucleotide DNAs: (Lanes 3 and 11) μE3; (lanes 4 and 12) μE3-mutant; (lane 5 and 13) USF; (lanes 6 and 14) μE3→USF). The bracket (left) identifies NF-μE3- (and possibly USF/MLTF−) binding activity.

Discussion

We describe here the isolation of a cDNA that encodes TFE3, a positive-acting ubiquitous transcription factor with binding specificity for the μE3 site of the IgH enhancer. The major open reading frame of the cDNA encodes a protein of 59 kD with several interesting putative structural motifs (Johnson and McKnight 1989; Mitchell and Tjian 1989). Among these are two motifs that have been shown for other proteins to facilitate protein–protein interactions. The MSR is thought to specify a helix–loop–helix structure and was initially described in MyoD, a protein involved in myogenic determination (Tapscott et al. 1988). It has now been implicated in a number of different proteins, including two additional myogenic factors, three other immunoglobulin enhancer-binding proteins, a gene associated with a chromosome translocation in a T-cell malignancy, and several *Drosophila* proteins (Mellentin et al. 1989; Murre et al. 1989a). For the immunoglobulin enhancer-binding proteins, which bind the μE5/κE2 motif, the region encompassing the helix–loop–helix has been shown to mediate DNA-binding through protein homodimer and heterodimer interactions (Murre et al. 1989a; Henthorn et al. 1990). Moreover, the MSRs of otherwise unrelated proteins (such as the κE2-binding protein E12 and MyoD or the *Drosophila daughterless* protein

product) can facilitate the formation of heterodimers that display enhanced DNA-binding affinities (Murre et al. 1989b). Hence, it is possible that the helix–loop–helix effectively acts as a regulatory domain and that *myc*-related helix–loop–helix proteins may modulate one another's activity. In addition to the helix–loop–helix, TFE3 contains a putative leucine zipper (Landschulz et al. 1988). The leucine zipper has been found in several transcription factors and has been shown in many cases to mediate function and/or protein–protein interactions, presumably via a coiled-coil structure (O'Shea et al. 1989). One of the more provocative examples of this involves the Fos–Jun interaction, for which both the Fos and Jun leucine zippers are required (Kouzarides and Ziff 1988, 1989; Sassone-Corsi et al. 1988; Gentz et al. 1989; Turner and Tjian 1989). It is possible that the TFE3 leucine zipper also mediates such heterodimer formation, or simply homodimer formation, as found with CCAAT/enhancer-binding protein (C/EBP) (Landschulz et al. 1989). Carboxy-terminal deletions of TFE3 that remove a single leucine reduce DNA-binding drastically (H. Beckmann and T. Kadesch, in prep.). Thus, the TFE3 zipper may indeed facilitate dimerization, if dimerization per se is required for DNA-binding. The location of the TFE3 leucine zipper adjacent to the helix–loop–helix, however, distinguishes it from other zippers that are often found next to basic regions. Perhaps this particular arrangement defines a new class of proteins whose ability to interact (either as homodimers or heterodimers) requires both motifs. The *myc* proteins may fall into this class, as they also possess leucine zippers.

TFE3 expressed from an SV40 early promoter was found to stimulate transcription in 3T3 cells from a pro-
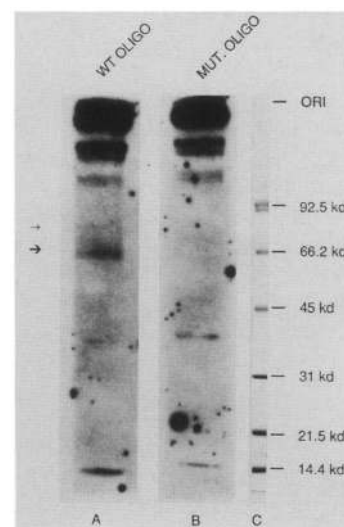

Figure 7. Identification of a μE3-binding activity, distinct from NF-μE3. Southwestern analysis of HeLa cell nuclear extracts was carried out as described in Methods. Membranes containing transferred proteins were exposed to μE3 (Lane A) or mutant-μE3 (Lane B) oligonucleotide probes. (Lane C) Stained molecular weight standards.

moter containing four copies of the μE3 site upstream of a TATA box. This confirms directly that TFE3 is a transcription factor. Cotransfected TFE3 also stimulated transcription in 3T3 cells from a transcription unit linked to a subfragment of the IgH enhancer (fragment 12), albeit to a lesser extent. The intact enhancer failed to respond to stimulation by TFE3 in 3T3 cells. Enhancer fragment 12 has been described previously as lacking a negative regulatory domain (Kadesch et al. 1986). Hence, this result is consistent with models that invoke negative regulation as a means of dictating B-cell-specific activity of the enhancer (Kadesch et al. 1986; Wasylyk and Wasylyk 1986; Weinberger et al. 1988). Presumably, the negative regulation works on the μE3 motif directly. The relatively low level of TFE3 *trans*-activation of enhancer fragment 12 compared to that of the [μE3]$_4$–TATA promoter may simply reflect a copy number effect. Enhancer fragment 12 carries only one copy of the μE3 site. Activation of a μE3–TATA promoter with only one μE3 site (as opposed to four) could not be detected in our TFE3 cotransfection experiments (H. Beckmann, L.-K. Su, and T. Kadesch, unpubl.).

We used fusions of GAL4 and TFE3 to map a strong activation domain in the TFE3 protein. It is located within the amino-terminal 126 amino acids of the protein, a region that is predicted to have some α-helical character and bears an overall net negative charge. These features have also been described for the activation domains of several transcription factors, including yeast GAL4 (Ma and Ptashne 1987) and GCN4 (Hope et al. 1988) and the herpesvirus VP16/Vmw65 (Cousens et al. 1989) and papillomavirus E2 proteins (Haugen et al. 1988; Lambert et al. 1989). Studies with model peptides support the idea that a negative amphipathic helix is sufficient for transcription activation (Giniger and Ptashne 1987). In fact, the strong activation domain of TFE3 contains a 12-amino-acid stretch that predicts such a helical structure. Our results also suggest the presence of a second, weaker, activation domain in the protein. We have not yet localized this domain to a specific region of TFE3. Considering what is presently known about transcription-activation domains (Mitchell and Tjian 1989), the proline/arginine-rich region at the carboxyl end would be considered a reasonable candidate (Mermod et al. 1989).

The presence of dimerization motifs in ubiquitous enhancer-binding proteins suggests an attractive model for cell-type-specific regulation. Perhaps TFE3 (and the μE5/κE2 binding proteins as well) functions as a ubiquitous DNA docking protein and transcriptional activation per se (or repression) is mediated through cell-type-specific proteins bound through their helix–loop–helix and/or leucine zipper motifs. This type of model is consistent with both genetic and physical evidence, which support the interplay between the *Drosophila* proteins *daughterless* and *achaete-scute*, both of which contain helix–loop–helix motifs (for further discussion, see Murre et al. 1989b and references therein). Our mapping of a strong activation domain in TFE3 outside the helix–loop–helix argues that transcriptional activation

per se is not mediated by a separate helix–loop–helix protein. However, it does not preclude models in which TFE3 is negatively regulated (masked?) by other helix–loop–helix proteins, perhaps bound at distinct sites on the enhancer.

We propose that TFE3 is not NF-μE3. This is based on the difference in the apparent sizes of the two proteins and on our identification of a μE3-binding protein in nuclear extracts that is distinct from NF-μE3. The open reading frame of the λ3 cDNA can encode a protein of 59 kD. If Met-41 is the true initiation codon, then TFE3 would be ~55 kD. Neither of these estimates is consistent with the sizes of the nuclear proteins that make up NF-μE3-binding activity (42.5–45 kD). This difference in size is also reflected by the slower mobility of TFE3 in mobility-shift assays. Moreover, we have detected a μE3-binding activity that is distinct from NFμE3 in nuclear extracts resolved with SDS-PAGE. The molecular weight of this activity (62–65 kD) is within a range consistent with its being TFE3. The data confirm that the experimental procedure can have a profound influence on the proteins one detects. It appears that NF-μE3 activity is sensitive to denaturation/renaturation whereas TFE3 activity is preferentially exposed by such treatment. We have confirmed that a 60- to 70-kD protein, and not a 42.5- to 45-kD protein, can be detected when HeLa nuclear proteins are resolved by SDS-PAGE, eluted from gel slices, and used in mobility-shift experiments with the μE3 oligonucleotide (H. Beckmann and T. Kadesch, unpubl.).

It is likely that NF-μE3 overlaps with or is equivalent to USF/MLTF. The latter activity has also been described as multiple 43- to 46-kD proteins (Chodosh et al. 1986; Sawadogo et al. 1988). Our experiments demonstrate that NF-μE3 binds very well to a USF/MLTF site, as does TFE3. We cannot say, however, whether TFE3 and USF/MLTF are related functionally because we have not yet been able to activate an adenovirus major late promoter in TFE3 cotransfection experiments (H. Beckmann, L.-K. Su, and T. Kadesch unpubl.). This raises the interesting possibility that TFE3 and USF/MLTF (or NF-μE3) display similar DNA binding specificities, yet activate transcription of distinct genes. This may provide an example similar to that found with the two octamer binding proteins OTF-1 and OTF-2 (Oct-1 and Oct-2). In this latter instance, the ability of each protein to activate transcription is thought to depend on distinct contexts with regard to additional transcription factors and other DNA binding proteins (Fletcher et al. 1987; Scheidereit et al. 1987; LeBowitz et al. 1988; Muller et al. 1988; Tanaka et al. 1988).

Methods

Oligonucleotide screening of phage library

A human B-cell-derived λgt11 cDNA library (Clontech) was screened with an μE3 oligonucleotide probe essentially as described by Vinson et al. (1988), with the following modifications. Probe consisted of a μE3 oligonucleotide (see below) end-labeled with [γ-^{32}P]ATP and then ligated to form random concatamers. Nitrocellulose filters bearing transferred phage were

denatured in buffer D [6 M guanidinium hydrochloride, 0.1 M KCl, 0.02 M HEPES (pH 7.9), 0.5 mM dithiothreitol (DTT)] and renatured in buffer R (buffer D minus 6 M guanidinium hydrochloride, plus 0.2 mM EDTA). After renaturation the filters were rinsed in buffer H [50 mM Tris-HCl (pH 7.5), 40 mM NaCl 1 mM EDTA, 1 mM DTT] and incubated at room temperature for 1 hr in buffer H supplemented with 5% nonfat dry milk. The filters were then washed twice in buffer B [10 mM Tris-HCl (pH 7.5), 40 mM NaCl, 1 mM EDTA, 1 mM DTT] and incubated for 1 hr in buffer B containing labeled oligonucleotide probe (5 × 10⁵ cpm/ml). Filters were washed for 30 min with four changes of buffer B and exposed to film.

DNA sequence analyses

Sequence analyses were performed on single-stranded DNA templates generated from the plasmid pBS-λ3 (see below) using the dideoxy chain-termination reaction (Sanger et al. 1977). The sequence was determined on a series of exonuclease III-generated subclones (Henikoff 1987) and by using specific internal primers. The coding region of the λ3 cDNA was sequenced on both strands.

Synthetic double-stranded oligonucleotides

The following synthetic oligonucleotides were synthesized by the University of Pennsylvania Cancer Center.

```
μE3:        5'-GATCTGGTCATGTGGCAAGGCTATTTGGG-3'
            3'-ACCAGTACACCGTTCCGATAAACCCCTAG-5'

μE3 mutant: 5'-GATCTGGTCTAGAGGCAAGGCTATTTGGG-3'
            3'-ACCAGATCTCCGTTCCGATAAACCCCTAG-5'

USF:        5'-GATCTGGTCACGTGGCCTACACCTATAAG-3'
            3'-ACCAGTGCACCGGATGTGGATATTCCTAG-3'

μE3 → USF:  5'-GATCTGGTCACGTGGCAAGGCTATTTGGG-3'
            3'-ACCAGTGCACCGTTCCGATAAACCCCTAG-5'
```

Plasmids

All plasmids and phage were constructed and manipulated using standard techniques (Maniatis et al. 1982). The 2500-bp λ3 cDNA was excised from phage λ3 DNA with *Eco*RI and inserted into the *Eco*RI site of pBluescript-SK(+) (Stratagene) to create pBS-λ3. The plasmid pSV2A-λ3 was constructed by excising the λ3 cDNA from pBS-λ3 with *Eco*RV and *Sma*I and using it to replace the luciferase gene between *Hin*dIII (filled) and *Sma*I of the plasmid pSV2ALΔ5' (De Wet et al. 1987). The plasmid pFBE3 was generated by ligating a *Bgl*I–*Hin*dIII fragment from p[E3]₄–TATA-CAT (containing the promoter), a *Hin*dIII–*Bgl*II fragment from pBS-λ3 (encoding the amino-terminal portion of the cDNA), and a *Bgl*II–*Bgl*I fragment from pSV2A-λ3 (encoding the carboxy-terminal portion of the λ3 cDNA and distal vector sequences). The plasmid containing the TATA box from the liver/bone/kidney alkaline phosphatase gene promoter linked to the bacterial CAT gene, TATA-CAT, and modifications carrying four copies of normal or mutant μE3 oligonucleotides [E3]₄–TATA-CAT and [mut-E3]₄–TATA-CAT, are described in detail elsewhere (M. Kiledjian and T. Kadesch, in prep.; D. Rueszinsky and T. Kadesch, in prep.). The reporter plasmids βG-CAT, βG-CAT-(2)₁₂, βG-CAT-(12)₁₂, and pSV2ACAT have been described previously (Kiledjian et al. 1988). All GAL4 fusion proteins were derived from the plasmid pGAL4₁₋₁₄₇ (Lillie and Green 1989). GAL4λ3 was constructed by inserting the λ3 cDNA (*Eco*RI–*Eco*RI) into the *Eco*RI site of pGAL4₁₋₁₄₇. GAL4λ3-Δ1 was generated by inserting a *Bgl*II–*Xba*I fragment from the λ3 cDNA into pGAL4₁₋₁₄₇ cut with

*Bam*HI and *Xba*I. GAL4λ3-Δ2 was created by inserting an *Eco*RI–*Bgl*II fragment from the λ3 cDNA into pGAL4₁₋₁₄₇ cleaved with *Eco*RI and *Bam*HI. In the plasmids GAL4λ3 and GAL4λ3-Δ1, the polylinker sequences lead to the insertion of amino acids Pro and Pro-Glu-Phe-Pro-Gly-Ile, between GAL4- and λ3-encoded amino acids, respectively. In GAL4λ3-Δ2, the polylinker leads to the insertion of Pro between GAL4- and λ3-encoded sequences and the amino acids Arg-Arg-Arg-Tyr-Pro-Gly-Glu-Phe-Glu-Leu at the carboxyl terminus. The plasmid βG-λ3 was constructed by ligating the *Eco*RV–*Xba*I fragment from pBS-λ3 (carrying the entire λ3 cDNA), an *Xba*I to *Bgl*I fragment from pGem4 (Promega) and a *Bgl*I–*Nco*I (filled) fragment from pT7βΔ-6Sal (Norman et al. 1988). This plasmid carries, in series, a T7 promoter, the 5'-leader and ATG from the human β-globin gene, one half of an *Eco*RV site (encoding an isoleucine), and the complete λ3 cDNA. Plasmids GAL4₁₋₁₄₇ and GAL4λ3-A, used for in vitro transcription and translation reactions (see Fig. 6), were constructed by inserting a *Hin*dIII–*Eco*RI fragment from pGAL4₁₋₁₄₇ between the *Hin*dIII and *Eco*RI sites of pGem3 (Promega) and a *Hin*dIII–*Taq*I fragment from GAL4λ3 between the *Hin*dIII and *Acc*I sites of pGem3.

Preparation of extracts

Nuclear extracts from HeLa and Namalwa cells were prepared as described by Dignam et al. (1983). Bacterial lysogens of λgt11 and λ3 were grown at 30°C to mid-log phase, induced for 30 min at 42°C, and grown for 60 min at 37°C in the presence of 10 mM IPTG. The pellet of a 1-ml culture was resuspended in 100 μl buffer D containing 0.5 mM PMSF (4°C), sonicated, and centrifuged (Eppendorf) for 15 min at 4°C. The supernatant was then dialyzed against buffer R containing 0.1% NP-40 for 6 hr at 4°C. Extracts were stored at −80°C.

Mobility-shift assays

Probes were prepared by labeling oligonucleotides or an IgH enhancer fragment either with T4 polynucleotide kinase (New England Biolabs) and [γ-³²P]ATP or by filling 5' overhangs with DNA polymerase (Klenow; Promega) and deoxynucleoside triphosphates containing [α-³²P]dCTP. Probes were separated from unincorporated nucleotides by gel electrophoresis (Maniatis et al. 1982). Enhancer fragment 12 was isolated from pSVAβG-CAT-(12)₁₂ (Kiledjian et al. 1988), as a *Xba*I–*Xba*I fragment. Binding reactions (0.015 ml) generally contained either 10 μl lysogen extract 10 μg nuclear extract, or 1 μl in vitro-translation reaction, 1 fmole double-stranded oligonucleotide (5000 cpm) or 1.3 fmole enhancer fragment (5000 cpm), 10 mM Tris-HCl (pH 7.5), 40 mM NaCl, 1 mM β-mercaptoethanol, 1 mM EDTA, and 4% glycerol. In addition, each reaction contained either 100 ng, 5 μg, or 500 ng poly[d(I-C)] (Pharmacia, Inc.) when lysogen extracts, nuclear extracts, or in vitro-translated proteins were assayed, respectively. For competition experiments, 1 pmole of unlabeled oligonucleotide was added to the binding reaction prior to the addition of extract. Electrophoresis was carried out using 5% polyacrylamide gels as described by Singh et al. (1988).

Methylation interference assays

For methylation interference assays, the 140-bp *Xba*I–*Eco*RI IgH enhancer fragment 20 (Kiledjian et al. 1988) was labeled at the *Xba*I site with T4 polynucleotide kinase and [γ-³²P]ATP for analysis of the upper strand (coding) or by filling the *Xba*I 5' overhang with DNA polymerase (Klenow) and [γ-³²P]dCTP for the lower strand (noncoding). The fragment was partially modified by DMS treatment (Maxam and Gilbert 1980) and incubated with 10 μl of in vitro-translated protein (GAL4λ3-A) in a

30-μl binding reaction containing 5 μg of poly[d(I-C)] for 30 min at room temperature. After electrophoresis on a nondenaturing gel, the retarded and free bands were treated essentially as described by Chodosh et al. (1988), using 8% denaturing polyacrylamide gels.

Transfections and CATase assays

Transfections of mouse NIH-3T3 cells were carried out by calcium phosphate coprecipitation as described by Graham and van der Eb (1973). The amounts of each plasmid used are indicated in the individual figures. Each transfection was carried out two to four times in independent experiments. Levels of β-galactosidase expression from the plasmid pCH110 (5 μg per transfection; Lee et al. 1984) were used to determine relative transfection efficiencies and to adjust the amount of extract used for the individual CATase assays (Gorman et al. 1982).

In vitro-transcription and translation reactions

In vitro transcription with T7 (Pharmacia) or Sp6 (Promega) RNA polymerase was performed with 1 μg linearized DNA templates, essentially as described by Melton et al. (1984). The resulting RNA was translated in a 50-μl volume containing 35 μl of rabbit reticulocyte lysate (Promega), as recommended by the manufacturer.

Southwestern analysis of nuclear proteins

HeLa cell nuclear extracts (0.5 mg per lane) were resolved by 10% SDS-PAGE, and proteins were electrophoretically transferred to a nitrocellulose membrane (Towbin et al. 1979). Membranes were then subjected to a cycle of denaturation and renaturation and exposed to oligonucleotide probes exactly as described above for λgt11 cDNA library screening. Standard protein molecular weight markers were obtained from Sigma.

Acknowledgments

Note added in proof

Recently Roeder et al. (pers. comm.) isolated a cDNA that potentially encodes USF/MLTF. The cDNA encodes a protein of roughly 40–45 kD, which binds to both the USF/MLTF site of the adenovirus major late promoter and the μE3 site of the IgH enhancer. Sequence comparison of their cDNA and that encoding TFE3 indicates that the two cDNAs are derived from distinct genes. Considering the likelihood that USF/MLTF and NF-μE3 represent the same DNA binding activity, the existence of their cDNA offers further support that TFE3 is not related to NF-μE3.

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