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Characterization of the transcription activation function and the DNA binding domain of transcriptional enhancer factor-1

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The regions of transcriptional enhancer factor-1 (TEF-1) required for its activation function and sequence-specific DNA binding have been determined. Deletion analysis of a chimera between TEF-1 and the GAL4 DNA binding domain (DBD) indicated that at least three regions of TEF-1 were involved in transactivation. However, none of these regions functioned as independent activating domains. Moreover, none of the GAL4 chimeras containing individual TEF-1 regions interfered with the activity of endogenous HeLa cell TEF-1, while interference was observed with the GAL4–TEF-1 chimeras which functioned as transactivators. These results indicate that there is a general correlation between the abilities of a given GAL4–TEF-1 chimera to function in transcriptional activation and interference, thus supporting the idea that transactivation by TEF-1 is mediated by a limiting transcriptional intermediary factor. In addition, we show experimentally that the TEA/ATTS domain is a novel class of DBD involved in the sequence-specific DNA binding of TEF-1 and its *Drosophila* homologue *scalloped*. Two other regions of TEF-1 are also required for DNA binding. These regions are not part of the minimum DBD, but may function by antagonizing the effect of sequences which negatively regulate DNA binding mediated by both the TEF-1 TEA/ATTS domain and the GAL4 DBD. In addition, analysis of TEF-1 and *scalloped* derivatives in which their TEA/ATTS domains have been interchanged further indicates that the TEA/ATTS domain is not the only determinant of DNA binding specificity.

Key words: Drosophila/SV40/TEA/ATTS domain/transcriptional interference

Introduction

Development and differentiation in eukaryotes result from spatially and temporally specific gene expression which is often controlled at the level of transcription initiation by the sequence-specific binding of transcriptional transactivators to their cognate *cis*-acting elements. Transcriptional activators comprise at least two functional features, the DNA binding domain (DBD) and the transcriptional activation function (AF), also referred to as the activating domain. AFs often exist as separable, interchangeable domains which can activate transcription both *in vivo* and *in vitro* when fused to a heterologous DBD. While no obvious sequence homology has been detected among the AFs of diverse transactivator proteins, AFs often have distinctive amino acid compositions. AFs which are particularly rich in acidic or hydroxylated (serine/threonine/tyrosine) amino acids, prolines or glutamines have previously been described (Hope and Struhl, 1986; Giniger and Ptashne, 1987; Ma and Ptashne, 1987; Courey and Tjian, 1988; Hollenberg and Evans, 1988; Triezenberg *et al.*, 1988; Mermod *et al.*, 1989; Theill *et al.*, 1989; Williams and Tjian, 1991; Seipel *et al.*, 1992; for reviews see Johnson and McKnight, 1989; Mitchell and Tjian, 1989; Ptashne and Gann, 1990; Carey, 1991, and references therein).

In contrast to the lack of sequence homology observed between AFs, the DBDs of many transactivators fall into readily identifiable classes. The members of each class exhibit partially conserved amino acid sequences, often corresponding to identifiable motifs, such as basic/leucine zippers, zinc fingers, helix–loop–helix, or homeo/POU domains (for reviews see Johnson and McKnight, 1989; Scott *et al.*, 1989; Berg, 1990; Hayashi and Scott, 1990; Brändén and Tooze, 1991; Harrison, 1991; Pabo and Sauer, 1992; and references therein), whose three dimensional structure has been (experimentally) determined. The structural motifs formed by other conserved classes of DBD such as the ets (Gutman and Wasylyk, 1990; Karim *et al.*, 1990; Thompson *et al.*, 1991; Wasylyk *et al.*, 1992, and references therein), rel (reviewed in Gilmore, 1990; Schmitz *et al.*, 1991; Blank *et al.*, 1992; Nolan and Baltimore, 1992) or MADS (Treisman and Ammerer, 1992) homologies are, however, as yet unknown. Moreover, other transcription factors such as the TATA binding protein (TBP) appear to have a unique type of DBD (Niklov *et al.*, 1992 and references therein). In addition to DNA binding, the conserved motifs in many classes of transactivator proteins also play a role in dimerization allowing the formation of homo- or heterodimers among the various members of a given family. This potential to form dimers vastly increases the combinatorial possibilities for gene regulation (for reviews see Jones, 1990; Kerr *et al.*, 1992; Leid *et al.*, 1992; Morrimoto, 1992 and references therein).

The mechanism by which transactivator proteins stimulate transcription is as yet unknown. *In vitro* studies using the chimeric acidic activator GAL–VP16 (Sadowski *et al.*, 1988; Triezenberg *et al.*, 1988; Cousens *et al.*, 1989; Carey *et al.*, 1990) have shown that this activator acts after the formation of template commited complexes to increase the number of active transcription complexes without notably increasing the rate of their formation (Wang *et al.*, 1992; White *et al.*, 1992). This effect may involve the direct interaction of the VP16 acidic AF with the general transcription factors, TBP (Stringer *et al.*, 1990; Ingles *et al.*, 1991) or TFIIB (Lin and Green, 1991). However, in common with other activators containing non-acidic AFs

such as Sp1, activation by VP16 *in vitro* also requires the presence of one or several transcriptional intermediary factors, also referred to as coactivators or mediators, which are required for activated but not basal transcription (Berger *et al.*, 1990; Kelleher *et al.*, 1990; Flanagan *et al.*, 1991; White *et al.*, 1991, 1992; Zhu and Prywes, 1992; Brou *et al.*, 1993a,b). Coactivators, such as 'USA' (Meisterernst *et al.*, 1991), may be chromatographically separable from the basal transcription factors, or they may be associated with TBP in the multiprotein TFIID complex (Peterson *et al.*, 1990; Dynlacht *et al.*, 1991; Tanese *et al.*, 1991; Takada *et al.*, 1992; Zhou *et al.*, 1992; Brou *et al.*, 1993a,b; for reviews see Pugh and Tjian, 1992; Gill and Tjian, 1992). Interestingly, the existence of coactivators or transcriptional intermediary factors (TIFs) required for activated but not basal transcription was implied by *in vivo* transcriptional interference ('squelching') experiments, where the overexpression of a transcriptional activator was shown to inhibit either its own activity (self-interference), or that of heterologous activators (Gill and Ptashne, 1988; Ptashne, 1988; Meyer *et al.*, 1989; Martin *et al.*, 1990; Ptashne and Gann, 1990; Tasset *et al.*, 1990; Krishna *et al.*, 1991; Martinez *et al.*, 1991). These *in vivo* studies suggested the existence of several distinct titratable TIFs with specificity for different classes of AF. However, the relationship between the TIFs identified by *in vivo* transcriptional interference experiments and the various coactivators required for activation *in vitro* is as yet unknown. Nevertheless, recent studies indicate that, in HeLa cell extracts, factors with the ability selectively to mediate transcriptional stimulation by activators with distinct AFs are associated with TBP in chromatographically separable TFIID complexes (Brou *et al.*, 1993a,b).

Transcriptional enhancer factor-1 (TEF-1) is a HeLa cell transactivator whose properties illustrate some of the principles discussed above. TEF-1 was first identified as a HeLa cell protein that binds cooperatively to tandem repeats of the GT-IIC or Sph enhansons from the simian virus 40 (SV40) enhancer (Davidson *et al.*, 1986, 1988; Wildeman *et al.*, 1986; Xiao *et al.*, 1987). These TEF-1 binding sites, which have highly degenerate nucleotide sequences, activate transcription from the SV40 early promoter (Davidson *et al.*, 1986; Herr and Clarke, 1986; Zenke *et al.*, 1986; Nomiyama *et al.*, 1987; Ondek *et al.*, 1987, 1988; Schirm *et al.*, 1987; Fromental *et al.*, 1988) and mediate large T antigen activation of the SV40 late promoter (May *et al.*, 1987; Casaz *et al.*, 1991; Gruda and Alwine, 1991; Kelly and Wildeman, 1991). The latter effect possibly involves direct interaction between TEF-1 and large T antigen (Gruda *et al.*, 1993). TEF-1 activity is not limited to the SV40 enhancer, since it also plays a role in expression from the human papilloma virus 16 upstream regulatory region (Ishiji *et al.*, 1992) and in that from a mutant of the polyoma virus enhancer (Herbomel *et al.*, 1984; Xiao *et al.*, 1987). In addition to viral regulatory elements, the avian homologue of TEF-1, M-CAT binding factor (MCBF), has been shown to be involved in muscle-specific gene expression (Farrance *et al.*, 1992), while the *Drosophila* homologue, *scalloped* (*sd*), is involved in the differentiation of the nervous system (Campbell *et al.*, 1992).

TEF-1 is encoded by a 426 amino acid open reading frame (ORF) initiated by an AUU codon (Xiao *et al.*, 1991). Analysis of the TEF-1 ORF indicated the presence of regions rich in acidic or hydroxylated (STY-rich) amino acids, as well as a proline-rich region and a region with the potential to form a zinc finger-like structure (Xiao *et al.*, 1991; see also Figure 1A). However, expression of recombinant TEF-1 in HeLa cells did not activate transcription from TEF-1 reporter genes to a level above that generated by the endogenous HeLa TEF-1, but rather repressed the activty of the endogenous HeLa TEF-1 (Xiao *et al.*, 1991). This dominant negative phenotype does not appear to require the site-specific binding of TEF-1 since it was also observed using chimeras in which the TEF-1 DBD had been replaced by that of the yeast activator GAL4 (Xiao *et al.*, 1991). In addition, low concentrations of a GAL4−TEF-1 chimera activated transcription from a GAL4 responsive reporter gene, but at high concentrations of this chimera the activation was repressed (Xiao *et al.*, 1991). These observations led us to propose that the TEF-1 AF required the action of a TIF(s) which was present in limiting amounts not only in HeLa cells but also in several other cell types (Ishiji *et al.*, 1992), and that the observed repression effects were due to transcriptional interference (Xiao *et al.*, 1991). Analogous results were also observed with full length recombinant TEF-1 and the GAL4−TEF-1 chimera in *in vitro* experiments using HeLa cell extracts (Xiao *et al.*, 1991).

We have previously proposed that the TEF-1 DBD was contained in a basic region located between amino acids 25 and 99 (Xiao *et al.*, 1991). This region of TEF-1 contains a novel putative conserved DBD called the TEA (Bürglin, 1991) or ATTS domain (Andrianopoulos and Timberlake, 1991), which is found also in the *Aspergillus abaA* gene product, the yeast transcription factor TEC-1, and *Drosophila sd* (Mirabito *et al.*, 1989; Laloux *et al.*, 1990; Campbell *et al.*, 1992). This novel domain is proposed to consist of either three α-helices, or one α-helix and two β-sheets, but the precise role of this domain in DNA binding has not been analysed experimentally.

In this study the regions of TEF-1 required for its activation function have been analysed. Maximal transactivation by TEF-1 involves the cooperation of the N-terminal acidic and/or proline rich-regions with the C-terminal region (residues 205−426). Nevertheless, none of the regions that contribute to transactivation by TEF-1 act as autonomous AFs when fused to a heterologous DBD, nor do they interfere with transactivation by the endogenous HeLa cell TEF-1. These results indicate that the individual regions identified above do not correspond to independent AFs which heterosynergize in wild-type TEF-1, but that they are simply constituents of a single AF. In addition, the role of the TEA/ATTS domain in sequence-specific DNA binding of the TEF-1 and *sd* proteins has been investigated. The TEA/ATTS domain alone is sufficient for specific DNA binding; mutation of two of its three predicted constituent α-helical and/or β-sheet structures shows that they are required for DNA binding. Thus, this domain is indeed a new class of DBD. Strikingly, however, analysis of TEF-1 C-terminal deletion mutants indicates that the STY-rich region and a C-terminal region are also required for DNA binding. These regions may function by counteracting the effect of a domain(s) that negatively regulates DNA binding mediated by the homologous TEF-1, and heterologous GAL4 DBDs. Our results also indicate that *sd* whose TEA/ATTS domain differs by only a single amino acid from that of TEF-1 does not efficiently bind *in vitro* to the GT-IIC and

Sph enhansons. Moreover, interchanging the TEF-1 and *sd* TEA/ATTS domains further indicates that this domain is not the sole determinant of sequence-specific binding.

Results

GAL4−TEF-1 chimeras stimulate transcription in HeLa cells

Transfection of HeLa cells with a vector expressing a chimeric fusion protein containing amino acids 2−426 of TEF-1 fused to the GAL4 DBD [GAL4(1−147)−TEF-1 (2−426) in Figure 1A] resulted in only a weak (2- to 3-fold) stimulation of expression from the GAL4 responsive UAS_G-tk-CAT reporter (Xiao *et al.*, 1991; also see lanes 3−6 in Figure 1B, summarized in Figure 1A). Surprisingly, this chimera containing the entire TEF-1 ORF was a weaker transactivator than a GAL4 chimera containing TEF-1 amino acids 167−426 [GAL4(1−147)−TEF-1(167−426) in Figure 3A and Xiao *et al.*, 1991] which stimulated expression 5- to 8-fold (lanes 10−13 in Figure 3B; see also Figure 3D and Xiao *et al.*, 1991). The low transactivation by the GAL4−TEF-1 (2−426) chimera cannot be explained by a lower expression of this protein, as Western blot analysis of transfected cell extracts using a mixture of two GAL4 monoclonal antibodies (see Materials and methods, and White *et al.*, 1992) shows that this chimera is efficiently expressed (lane 2, Figure 1C). In addition, analysis of transfected cell nuclear extracts by electrophoretic mobility shift assay (EMSA) indicated that both of these chimeras formed complexes with oligonucleotides containing a wild-type consensus 17mer GAL4 binding site, but not with those containing a mutated one (compare lanes 3−4 and 13−14 with lanes 21−22 containing a control extract in Figure 2A).

It has been reported that GAL4−AP-2 and CTF/NF-1-Sp1-DBD chimeras, which comprise more than one functional DBD, are weak transactivators (Mermod *et al.*, 1989; Williams and Tjian, 1991) although they contain strong AFs. To test the possibility that the effect of a strong AF in the chimera containing the total TEF-1 ORF was being underestimated by the artefactual presence of two DBDs, a chimera was constucted from which the TEF-1 DBD had been deleted [GAL4(1−147)−TEF-1Δ55−121 in Figure 1A]. This chimera had no effect on expression from the pBLCAT8+ reporter lacking the GAL4 binding sites (see lane 1 in Figure 1B), but exhibited much stronger (maximally 60-fold) transactivation activity than GAL4−TEF-1(2−426) with the UAS_G-tk-CAT reporter (compare lanes 7−10 with lanes 3−6 in Figure 1B; summarized in Figure 3D). Thus, deletion of amino acids 55−121 leads to a large increase in transactivation possibly due to the deletion of the TEF-1 DBD. Note also that, as previously observed (Xiao *et al.*, 1991), transfection of higher concentrations of expression vector resulted in a decrease in transactivation activity (see Figure 3D).

Analysis of the TEF-1 ORF indicated the presence of regions which are particularly rich in certain amino acids, such as acidic residues, proline or serine/threonine/tyrosine (STY-rich) (see Figure 1A). In addition, the C-terminal 34 amino acids structurally resemble the zinc-finger motif ($-CX_2CX_8HX_3H-$) of transcription factor TFIIIA from *Xenopus laevis* (Klug and Rhodes, 1987) and have the potential to form an α-helix. To test the possible contribution of the C-terminal and STY-rich regions to transactivation

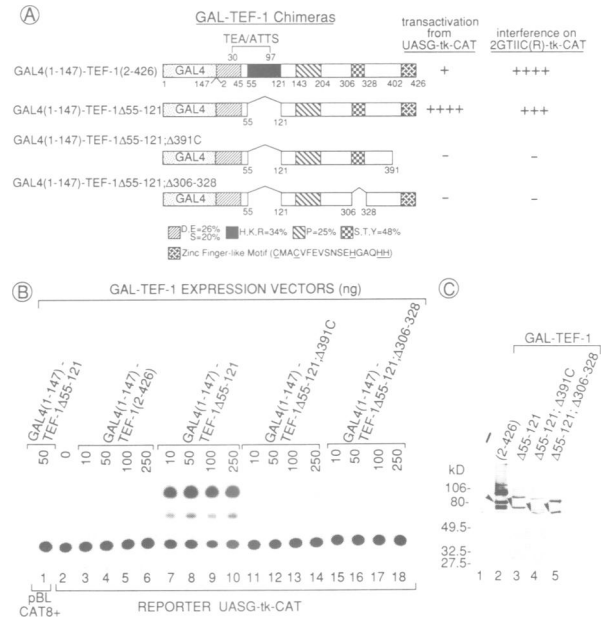

Fig. 1. (**A**) Structural features of the TEF-1 amino acid sequence and the structures of the GAL4−TEF-1 chimeras. The first line shows the structure of a GAL4−TEF-1 chimera in which the entire TEF-1 ORF (amino acids 2−426) is fused to the GAL4 N-terminal amino acids 1−147. The regions of TEF-1 with distinctive amino acid compositions are indicated by the hatched boxes, and the position of the TEA/ATTS domain is indicated. The numbers above and below refer to the TEF-1 or GAL4 amino acids. The amino acid compositions are given using the single letter code. The ability of the chimeras to stimulate transcription from the UAS_G-tk-CAT reporter, or to interfere with the activity of the endogenous HeLa cell TEF-1 on the 2GTIIC(R)-tk-CAT reporter are qualitatively summarized on the right of the figure. The activity of the most active chimera in each assay is taken as 100% (++++). (**B**) The autoradiographic image of representative CAT assays using extracts from transiently transfected HeLa cells is shown. The quantities (in ng) of the GAL4−TEF-1 expression vectors used in each transfection are indicated above each lane, and the reporter plasmid below. (**C**) Western blot analysis of nuclear extracts from HeLa cells transfected with 10 μg of the expression vectors for the chimeras, indicated above each lane, is shown. 10 μg of protein from each extract was separated by SDS−PAGE and transferred to nitrocellulose. The GAL4−TEF-1 chimeras were revealed using anti-GAL4 monoclonal antibodies. The positions of the full-length chimeras are indicated by the arrows. Lane 1 contains a control extract from cells transfected with the empty pXJ40 expression vector. The positions of migration of molecular weight standards with the indicated relative molecular masses (in kDa) are shown to the left of the figure.

by TEF-1, each region was deleted in the context of the Δ55−121 chimera [mutants GAL4(1−147)−TEF-1Δ55−121;Δ391C, and GAL4(1−147)−TEF-1Δ55−121;Δ306−328 respectively in Figure 1A]. Interestingly, deletion of the STY-rich region completely abolished the ability of the corresponding chimera to stimulate expression from the UAS_G-tk-CAT reporter, even when up to 1 μg of the expression vector was transfected, while the chimera deleted in the C-terminal region activated transcription weakly only at the highest concentrations (3-fold with 1 μg, see lanes 11−18 in Figure 1B, summarized in Figure 3D, and data not shown). The expression and nuclear localization of these chimeras were verified by Western blot using nuclear extracts from transfected cells (see Figure 1C) and immunofluorescence (data not shown). The levels of expression of GAL4−TEF-1Δ55−121;Δ391C and GAL4−TEF-1Δ55−

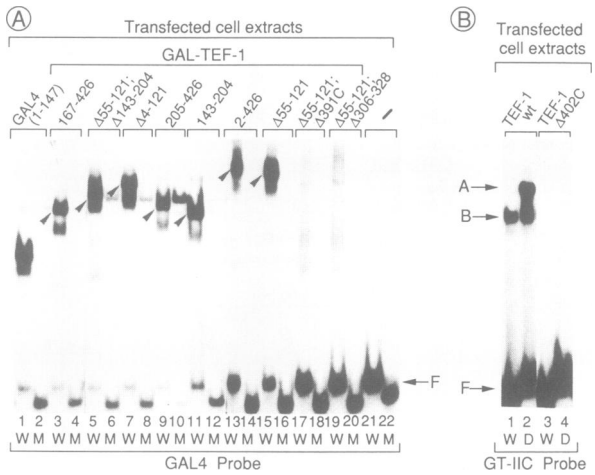

Fig. 2. (**A**) Analysis of transfected cell nuclear extracts by EMSA. The ability of the GAL4−TEF-1 chimeras present in the transfected cell nuclear extracts analysed by Western blotting in Figures 1C and 3C to bind to a perfectly palindromic 17mer GAL4 binding site was determined in an EMSA. The transfected GAL4−TEF-1 chimeras are indicated above each lane. Lanes 21−22 contain an extract from HeLa cells transfected with the empty expression vector pXJ40. W and M indicate the use of a wild-type or mutated GAL4 binding site. The positions of the specific complexes are indicated by the arrows. (**B**) The ability of the wild-type TEF-1 or the TEF-1Δ402C proteins, present in the transfected cell nuclear extracts analysed by Western blotting in Figure 5D, to bind to the GT-IIC enhanson was determined by EMSA. The position of the B and A complexes generated by the binding of TEF-1 to a single or tandemly repeated GT-IIC enhanson respectively, as previously described (Davidson *et al.*, 1988; Xiao *et al.*, 1991), are indicated to the left of the panel. W and D indicate oligonucleotides containing a single GT-IIC enhanson or a tandemly repeated (dimer) GT-IIC enhanson, respectively.

121;Δ306−328 differed by no more than 2- to 3-fold compared with GAL4−TEF-1Δ55−121 (compare lanes 3−5 in Figure 1C), while they were unable to activate transcription efficiently when transfected over a 10 ng−1 μg (100-fold) concentration range. Thus, the inability of these deletion mutants to transactivate cannot be attributed to the absence or instability of the corresponding chimeric proteins in the nuclei of transfected cells.

The expression of the chimeras in nuclear extracts from transfected cells was also determined by EMSA using oligonucleotides containing a wild-type or mutated GAL4 binding site. Specific complexes were efficiently formed with the GAL4−TEF-1(167−426), (2−426) and Δ55−121 chimeras, but surprisingly no such complexes could be detected with GAL4−TEF-1Δ55−121;Δ391C or ;Δ306−328 (lanes 3−4 and 13−20, Figure 2A) despite the fact that the chimeras could readily be detected in the transfected cell nuclear extracts (Figure 1C). In each case a smear was detected rather than a discrete complex, suggesting that the complexes formed by these two chimeras had a reduced stability. Thus, as deletion of these two TEF-1 regions changes the DNA binding properties of the corresponding GAL4−TEF-1 chimeras (see also below), no conclusions concerning the contribution of these two regions to the AF can be drawn from these transactivation assays.

Transactivation by TEF-1 requires cooperation between the acidic N-terminal and/or proline-rich regions and the C-terminal 205−426 amino acids

The role of the acidic N-terminal or proline-rich regions (amino acids 4−54 and 143−204, respectively, see Figures 1A and 3A) in transactivation by TEF-1 were next investigated. GAL4−TEF-1Δ55−121 chimeras from which only one of the above regions had been deleted [mutants GAL4(1−147)−TEF-1Δ4−121 and GAL4(1−147)−TEF-1Δ55−121;Δ143−204 lacking the acidic or proline-rich regions respectively, see Figure 3A], but which contain the STY-rich and C-terminal regions, stimulated expression from UAS_G-tk-CAT 2- to 3-fold less efficiently than GAL4−TEF-1Δ55−121, which contains all four regions (lanes 2−9 in Figure 3B and lanes 7−10 in Figure 1B; summarized in Figure 3D). As described above, the chimera GAL4−TEF-1(167−426), in which both the acidic region and the N-terminal portion of the proline-rich region were deleted, was a weaker transactivator than GAL4−TEF-1Δ4−121 containing the entire proline-rich and flanking regions (see Figure 3B, lanes 10−13, and Figure 3D). Furthermore, total deletion of both the acidic and proline-rich regions [mutant GAL4(1−147)−TEF-1(205−426), see Figure 3A and lanes 14−17 in Figure 3B] completely abolished transactivation. The above results show that the acidic and/or the proline-rich regions can cooperate with the C-terminal 205−426 region to allow transactivation, but that maximum activity requires the presence of all three regions.

Each of the above regions of TEF-1 was then fused, individually or in combination, with the GAL4 DBD to determine whether they contained autonomously acting AFs capable of stimulating expression from the UAS_G-tk-CAT reporter (see Figure 3A). Chimeras containing either the acidic, the proline-rich or the C-terminal regions [mutants GAL4(1−147)−TEF-1(2−45), GAL4(1−147)−TEF-1(143−204) and GAL4(1−147)−TEF-1(328−426) respectively in Figure 3A] did not stimulate transcription following transfection in HeLa cells (summarized in Figure 3A and data not shown). These results indicate that none of these regions on their own contain an AF. Furthermore, as described above, the GAL4−TEF-1(205−426) chimera containing both the STY-rich and C-terminal regions, but lacking the acidic or proline rich-regions, did not function as a transactivator (see lanes 14−17 in Figure 3B).

The expression of the chimeric proteins in transfected cells was verified by Western blot analysis using the monoclonal anti-GAL4 antibodies and EMSA. As each of the inactive chimeras was expressed at levels comparable to (or higher than) those which function as transactivators (compare lanes 3−4 with lanes 2 and 5−7 in Figure 3C; and data not shown), the lack of activity of these chimeras, transfected over a 25-fold concentration range, cannot be ascribed to differences in the levels of expressed protein. In addition, each of these chimeras formed specific complexes with the GAL4 binding site in EMSA (lanes 3−12, Figure 2 and data not shown). These results indicate that in HeLa cells, in this promoter context, none of the above TEF-1 regions contain autonomous AFs although they contribute to transactivation by the GAL4−TEF-1 chimera. Even the chimera GAL4−TEF-1(205−426), which contains the combination of the STY-rich and C-terminal regions, did not function as a transactivator in this context.

Regions of TEF-1 required for transactivation are also required for self-interference

Ectopic expression of wild-type TEF-1 or the GAL4−TEF-1(167−426) chimera in HeLa cells leads to

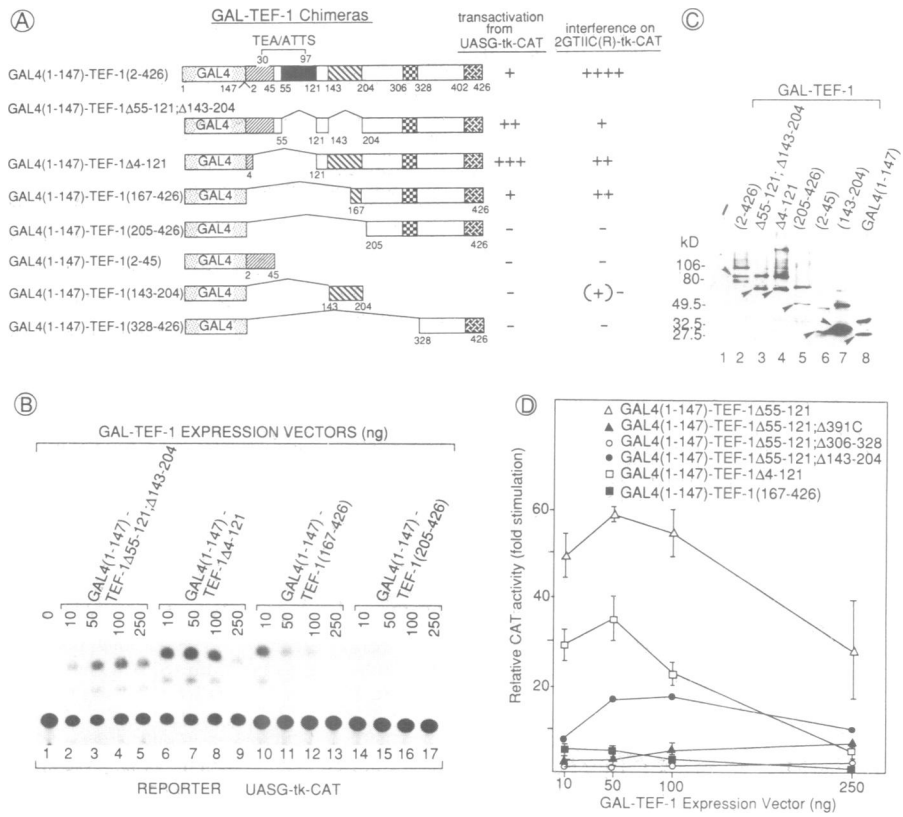

Fig. 3. (**A**) Structure of GAL4−TEF-1 chimeras. The nomenclature used is as described in Figure 1A. (**B**) The autoradiographic image of representative CAT assays using extracts from transfected HeLa cells is shown. The nomenclature is as described in Figure 1B. (**C**) The presence of the GAL4−TEF-1 chimeras in the nuclear extracts from HeLa cells transfected with the expression vectors indicated above each lane was detected by Western blot analysis using the anti-GAL4 monoclonal antibodies. The positions of the full length chimeras are indicated by the arrows. Lane 1 contains a control extract from cells transfected with the empty pXJ40 expression vector. The nomenclature is as described in Figure 1C. (**D**) The relative abilities of the GAL4−TEF-1 chimeras to stimulate transcription from the UAS_G-tk-CAT reporter are graphically represented. The values represent the average of two independent transfections in the case of GAL4−TEF-1Δ55−121;Δ143−204. In all other cases, the values represent the average ± standard deviation, of three or more independent transfections using at least two independently isolated clones.

a reduction in the activity of the endogenous HeLa TEF-1, as indicated by a decrease in the expression from the 2GTIIC(R)-tk-CAT reporter containing eight TEF-1 binding sites upstream of the tk promoter (Xiao *et al.*, 1991; Ishiji *et al.*, 1992). We have previously proposed that this dominant negative phenotype was due to transcriptional self-interference (see Introduction). To explore the relationship between the domains of TEF-1 required for transcriptional activation and self-interference, the ability of the GAL4−TEF-1 chimeras to stimulate expression from UAS_G-tk-CAT was compared with their ability to interfere with the activity of the endogenous HeLa TEF-1 using the 2GTIIC(R)-tk-CAT reporter. GAL4−TEF-1Δ55−121, which exhibited the strongest transactivation activity, inhibited expression from the 2GTIIC(R)-tk-CAT reporter more efficiently than GAL4−TEF-1Δ4−121 and GAL4−TEF-1Δ55−121;Δ143−204, which were weaker transactivators (see Figure 4). Mutants lacking the C-terminus (GAL4−TEF-1Δ55−121;Δ391C) or the STY-rich region (GAL4−TEF-1Δ55−121;Δ306−328), which did not efficiently transactivate, were also unable to interfere with the activity of the endogenous TEF-1 (see Figures 1A and 4). Similarly, the GAL4−TEF-1 chimeras containing only the N-terminal acidic, or proline-rich regions, or the C-terminal 205−426 region, which did not transactivate, were also unable to efficiently interfere with the activity of endogenous TEF-1 (data not shown, summarized in Figure 3A). These results indicate that there is a general correlation between the efficiencies of transactivation and interference with the activity of endogenous TEF-1 by a given chimera. Nevertheless, interference by GAL4−TEF-1(167−426) was more efficient than that by GAL4−TEF-1Δ55−121;Δ143−204, which was a stronger transactivator. Furthermore, GAL4−TEF-1(2−426), which was only a weak transactivator, interfered with endogenous TEF-1 activity almost as efficiently as the full length wild-type TEF-1, and thus, more efficiently than GAL4−TEF-1Δ55−121, which was the strongest activator (compare Figures 4 and 5C).

A series of C-terminal deletions and a deletion of the TEF-1 DBD were also constructed in the context of wild-type TEF-1 (see Figure 5A), and each of the mutants was examined for its ability to interfere with the activity of endogenous TEF-1 by transfection in HeLa cells. Endogenous HeLa cell TEF-1 stimulated expression from the 2GTIIC(R)-tk-CAT reporter >10-fold compared with the enhancerless pBLCAT8+ construct (compare lanes 2 and 24 with lane 1 in Figure 5B). As previously reported (Xiao *et al.*, 1991), transfection of nanogram amounts of a vector expressing wild-type TEF-1 efficiently inhibited expression from the 2GTIIC(R)-tk-CAT reporter (compare lanes 2 and 24 and lanes 3−6 in Figures 5B and C). Transfection of a vector expressing TEF-1Δ55−121, from which the putative DBD (see also below) was deleted, also

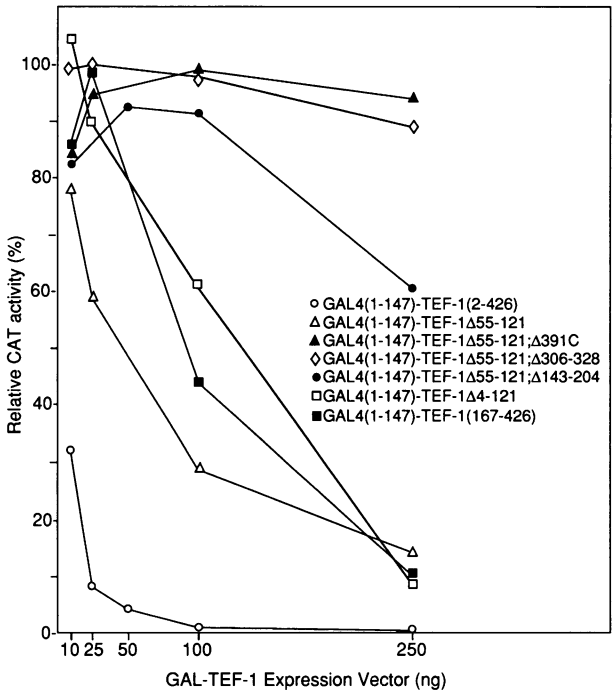

Fig. 4. The abilities of the GAL4−TEF-1 chimeras to interfere with the activity of the endogenous HeLa cell TEF-1 are graphically represented. The activity of the 2GTIIC(R)-tk-CAT reporter in the absence of transfected GAL4−TEF-1 chimeras, but in the presence of the pXJ40 expression vector is taken as 100%. The values represent the average of at least three independent transfections using at least two independently isolated clones.

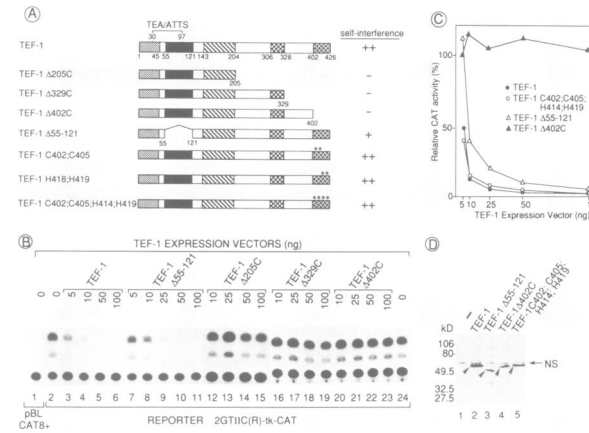

Fig. 5. (**A**) The structural features of the TEF-1 amino acid sequence are represented as in Figure 1A. The ability of the transfected wild-type or mutated TEF-1 proteins to interfere with the activity of the endogenous HeLa cell TEF-1 on the 2GTIIC(R)-tk-CAT reporter is qualitatively summarized to the right. The locations of the mutated cysteine and histidine residues are indicated by asterisks. (**B**) Representative CAT assays using extracts from HeLa cells transfected with the expression vectors indicated above each lane are shown. The nomenclature is as in Figure 1B. (**C**) The ability of the wild-type and mutated TEF-1 proteins to interfere with the activity of the endogenous HeLa cell TEF-1 is graphically represented. The activity of the 2GTIIC(R)-tk-CAT reporter in the absence of ectopically expressed TEF-1, but in the presence of pXJ40, is taken as 100%. The values represent the averages of at least three independent transfections using at least two independently isolated clones. (**D**) The presence of the wild-type and mutated TEF-1 proteins in nuclear extracts from transfected cells was detected by Western blotting using the anti-P1 serum recognizing TEF-1 amino acids 1−11 (Xiao *et al.*, 1991). The positions of the recombinant proteins are indicated by arrows. NS indicates the presence of a HeLa cell protein that is non-specifically recognized by the antiserum. Lane 1 contains a control extract from cells transfected with the empty pXJ40 expression vector.

inhibited expression from the 2GTIIC(R)-tk-CAT reporter, albeit less efficiently than wild-type TEF-1 (compare lanes 7−11 with lanes 3−6 in Figure 5B, see also Figure 5C). In agreement with the result previously obtained using GAL4−TEF-1(167−426) (Xiao *et al.*, 1991), this result clearly confirms that site-specific DNA binding by TEF-1 is not required for the dominant negative phenotype.

In addition to the TEF-1Δ205C and TEF-1Δ329C mutants [in which large regions were deleted from the C-terminus (see Figure 5A)], mutant TEF-1Δ402C, in which only 23 C-terminal amino acids were deleted, was unable to interfere with the activity of endogenous HeLa cell TEF-1 (see lanes 12−23 in Figure 5B). As described above, this C-terminal region contains two cysteines and three histidines which resemble a zinc-finger motif (see Figure 1A and Xiao *et al.*, 1991). However, mutation of these cysteines and/or histidines to alanine (mutants TEF-1C402;C405, TEF-1H418;H419 and TEF-1C402;C405;H414;H419 in Figure 5A) did not affect the ability of the corresponding proteins to interfere with the activity of endogenous TEF-1 (see Figure 5C, and data not shown). Similarly, introduction of the double cysteine/histidine to alanine mutations in the GAL4−TEF-1(2−426) chimera had no effect on its ability to interfere with the endogenous HeLa TEF-1, while the equivalent chimera with the Δ402C deletion was unable to interfere (see Figure 5C, and data not shown).

Western blot analysis of extracts from transfected cells using the anti-P1 TEF-1 antiserum (see Materials and methods and Xiao *et al.*, 1991) indicated that the wild-type and mutant proteins were expressed at comparable levels (compare lanes 2−5 in Figure 5D, and data not shown). In addition, the nuclear localization of the wild-type and Δ402C mutant was verified by immunofluoresence (data not shown).

The TEA/ATTS domain of TEF-1 is necessary and sufficient to determine sequence-specific binding to both the SV40 GT-IIC and Sph enhansons, but DNA binding is modulated by other regions of TEF-1

We have previously suggested that the TEF-1 DBD was located between amino acids 25 and 99 (Xiao *et al.*, 1991). This region contains a novel putative class of DBD called the TEA (Bürglin, 1991) or ATTS (Andrianopoulos and Timberlake, 1991) domain (located between amino acids 30 and 97/101), which is predicted to consist of either three α-helices or one α-helical and two β-sheet structures (Bürglin, 1991; Andrianopoulos and Timberlake, 1991; Campbell *et al.*, 1992; see also Figure 6A). Proline residues were introduced at highly conserved positions in each of the three putative α-helices (see * in Figure 6A) to determine the role of these regions and their potential α-helicity in sequence-specific DNA binding. The ability of TEF-1 containing the mutated TEA/ATTS domains to bind to the GT-IIC and Sph enhansons was examined by EMSA following transcription and translation *in vitro*. In addition, in order to take advantage of the strikingly high sequence homology in the TEA/ATTS domain between the TEF-1 and *sd* proteins (Figure 6A), the *sd* coding region was subcloned in the pXJ40 vector to allow transcription and translation *in vitro*. The wild-type and mutated proteins were all

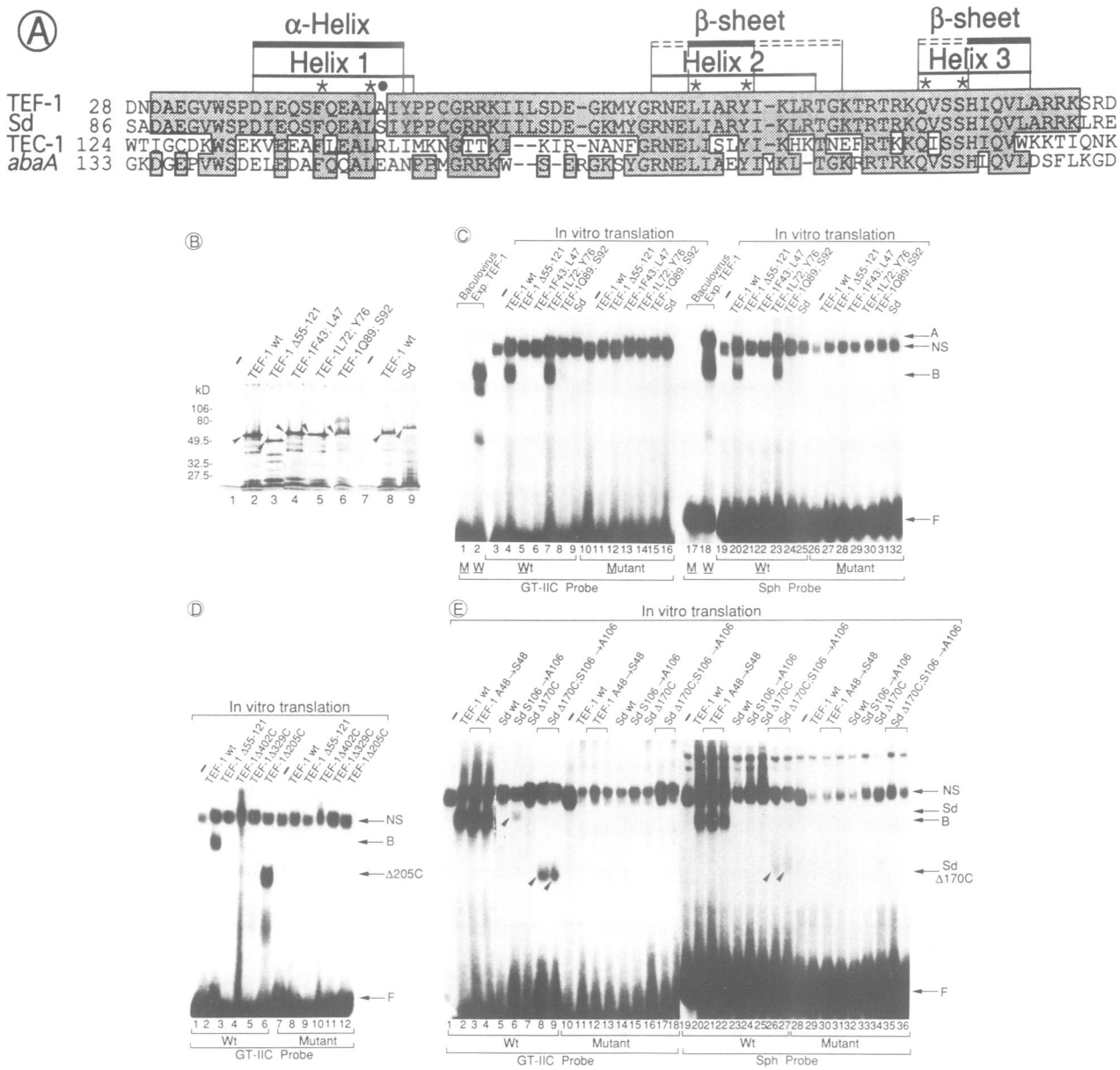

Fig. 6. (**A**) The amino acid sequences of the TEA/ATTS domains from the TEF-1, *sd*, TEC-1 and *abaA* gene products are shown. The conserved amino acids are in the shaded boxes. The numbers to the left of each line are the numbers of the first amino acid of the TEA/ATTS domain in each protein. The locations of the predicted α-helical and or β-sheet structures (Bürglin, 1991; Adrianopoulos and Timberlake, 1991) are indicated above the TEF-1 sequence. The locations of the conserved amino acids which have been mutated to proline are indicated by asterisks, while the single amino acid change between the TEF-1 and *sd* sequences is indicated by ●. (**B**) A representative experiment showing the production of the wild-type and mutated TEF-1 and *sd* proteins (indicated above each lane) by transcription and translation *in vitro* is presented. The ^{35}S-labelled proteins were detected by autoradiography and their positions are indicated by arrows. The preparations shown in this experiment were used for the EMSAs shown in panel C. The preparations of the proteins used in panels D and E were verified in the same way (data not shown). (**C**, **D** and **E**) The binding of the wild-type and mutated TEF-1 and *sd* proteins, generated by transcription and translation *in vitro*, to the GT-IIC and Sph enhansons was determined by EMSA. The proteins used are indicated above each lane. NS indicates the position of a non-specific complex generated using all the proteins including the control reticulocyte lysate without exogenously added RNA (e.g. lane 3, panel C). The positions of the specific A and B complexes generated by *in vitro* translated or baculovirus expressed TEF-1 are also indicated. F is the free DNA. The arrows within the figure indicate the positions of the complexes generated by the full length and truncated *sd* proteins.

produced in comparable amounts (see Figure 6B). Equivalent amounts of each protein were analyzed for their abilities to bind to the GT-IIC and Sph enhansons using EMSA. The wild-type TEF-1 protein produced either by *in vitro* translation or in a baculovirus expression system bound the wild-type GT-IIC and Sph enhanson probes but not the mutated ones (compare lanes 1 and 2, 4 and 11, 17 and 18, and 20 and 27 in Figure 6C). TEF-1Δ55−121, in which most of the TEA/ATTS domain was deleted, was unable to bind to either the GT-IIC or Sph enhansons (see lanes 5, 12, 21 and 28 in Figure 6C, and lanes 3 and 9 in Figure 6D). Interestingly, mutation of the first putative α-helical structure completely abolished binding to both enhansons (see lanes 6, 13, 22 and 29 in Figure 6C). In contrast, mutation of the second putative α-helical structure appeared to have no effect on DNA binding (compare lanes 7 and 23 with lanes 14 and 30, respectively in Figure 6C). The introduction of prolines into the third putative α-helix

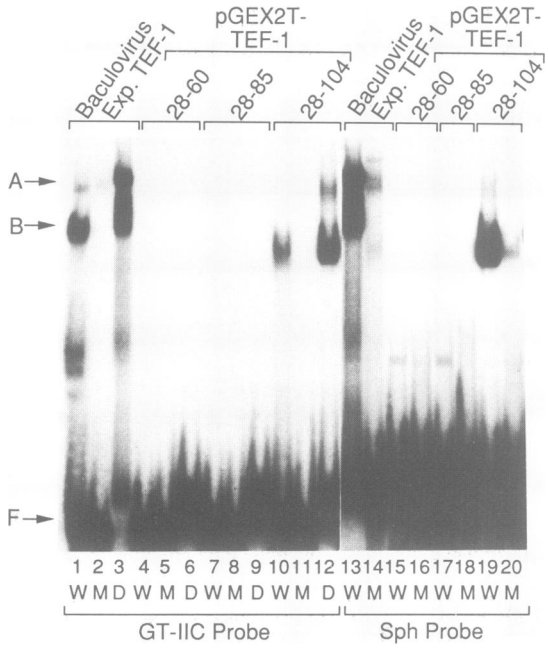

Fig. 7. The binding of the GST−TEF-1 fusion proteins to the GT-IIC and Sph enhansons was determined by EMSA. The proteins used are indicated above each lane. The positions of the A and B complexes generated by the full length recombinant baculovirus expressed TEF-1 are indicated. F is the free DNA. W and M are the wild-type or mutated GT-IIC or Sph enhansons, while D is the tandemly repeated GT-IIC enhanson.

resulted in weak but detectable binding to both enhansons (see lanes 8 and 24 in Figure 6C). Strikingly, no binding to either the wild-type or mutated GT-IIC or Sph enhansons was detected using the *sd* protein (lanes 9, 16, 25 and 32 in Figure 6C, and lanes 5, 14, 23 and 32 in Figure 6E), even when excess amounts of protein were used (data not shown).

The above results indicate that the first and third α-helix/β-sheet of the TEA/ATTS domain are required for sequence-specific DNA binding *in vitro*. Surprisingly, however, no binding to the GT-IIC or Sph enhansons was observed with the *in vitro* translated TEF-1 C-terminal deletion mutants Δ402C and Δ329C (lanes 4−5 and 10−11, Figure 6D and data not shown), which contain an intact TEA/ATTS domain. Similar results were obtained using transfected cell extracts (Figure 2B, and data not shown). In contrast, specific binding was observed using Δ205C (lanes 6 and 12 Figure 6D, and data not shown). These results, together with those described above using the GAL4−TEF-1 chimeras (Figure 2A), suggest that the C-terminal region contains sequences which may both positively and/or negatively modulate DNA binding.

In view of the above results it was necessary to determine whether the TEF-1 TEA/ATTS domain alone was sufficient for sequence-specific binding, or whether a combination of the C-terminal and TEA/ATTS regions was required. The TEF-1 amino acids encoding α-helical structure 1 alone or a combination of α-helices/β-sheets 1+2 or 1+2+3, were fused to the glutathione-S-transferase (GST) gene in plasmid pGEX2T [pGEX2T−TEF-1(28−60), (28−85) or (28−104) respectively]. Extracts from IPTG-induced *Escherichia coli* harbouring these plasmids were then used in EMSA. No specific binding to the GT-IIC or Sph enhansons was detected using the GST derivatives containing α-helix 1 alone or the combination of helices 1+2 (lanes 4−9 and 15−18 in Figure 7). Specific binding to the wild-type but not to the mutated GT-IIC and Sph enhansons was, however, detected with the combination of helices 1+2+3 (lanes 10−12 and 19−20, Figure 7). These results indicate that the TEF-1 TEA/ATTS domain alone is necessary and sufficient to direct sequence-specific binding.

As the TEA/ATTS domain alone is sufficient to allow specific DNA binding, the inability of the *sd* TEA/ATTS domain to bind the GT-IIC and Sph enhansons in this *in vitro* assay suggests that the first α-helical structure of the TEA/ATTS domain may be functionally equivalent to the recognition helix 3 of the homeodomain (Kissinger *et al.*, 1990; Wolberger *et al.*, 1991), and thus play a role in determining the precise DNA sequence recognized by a given TEA/ATTS domain. To test this hypothesis alanine 48 of TEF-1 was mutated to serine as in *sd* and vice versa (mutants TEF-1 A48-S48, and *sd* S106-A106), and the mutated proteins were produced by transcription and translation *in vitro*. Weak but detectable binding of the *sd* S106-A106 mutant protein to the wild-type but not the mutated GT-IIC enhansons was observed (lanes 6 and 15 in Figure 6E). However, no binding of this mutated *sd* protein to the Sph enhansons was detected even after prolonged exposure of the autoradiogram (lanes 24 and 33 in Figure 6E, and data not shown). Similarly, no binding to the wild-type GT-IIC or Sph enhansons was detected using a C-terminally truncated *sd* protein containing a wild-type TEA/ATTS domain (*sd* Δ170C in lanes 7 and 25 in Figure 6E). Interestingly, in the context of *sd* Δ170C introduction of the S106-A106 mutation allowed specific binding not only to the GT-IIC enhanson, as in the full length *sd* protein, but also to the Sph enhansons (lanes 8−9, 17−18, 26−27 and 35−36 in Figure 6E). Surprisingly, however, in the context of TEF-1 the A48-S48 mutation appeared to have no effect on the binding of TEF-1 to the GT-IIC or Sph enhansons (lanes 2−4 and 20−22 in Figure 6E). Thus, these different context-specific effects of the A→S mutation in the first α-helical structure of the TEA/ATTS domain suggest that, while it plays a role in sequence-specific DNA binding, it is not the sole determinant of specificity.

Discussion

Three regions of TEF-1 contribute to transcriptional activation and interference

The analysis of many transcriptional activators has shown that they often have a modular organization (see Introduction for references). For example, deletions in the glutamine-rich regions of Sp1, or in the A/B or E regions of members of the steroid hormone receptor family, have defined domains containing AFs that also activate transcription when fused to heterologous DBDs (see Green and Chambon, 1988; Mitchell and Tjian, 1989; Gronemeyer, 1991, and references therein). In some transcription factors, such as Sp1 or Oct(OTF)-2, the transcriptional activating domains appear to be at least partially redundant (Courey and Tjian, 1988; Müller-Immerglück *et al.*, 1990), while in others, for example Myf-5, the cooperation of at least two activating domains is absolutely required (Winter *et al.*, 1992). The results of the present study show that at least three regions of TEF-1 contribute to its AF, but that none of these regions

functioned as autonomous activating domains when fused to a heterologous DBD. In addition, none of these regions, on their own, interfere with transactivation by endogenous HeLa cell TEF-1, further indicating that they are not independent activating domains which heterosynergize in the context of wild-type TEF-1. One of the most striking observations, however, is that all of the deletions have an effect on transcriptional activation and/or interference. This suggests either that sequences contributing to these functions may be dispersed throughout the entire protein, or that a precise spacing and/or orientation of the different elements is required for their optimal function.

A GAL4 chimera containing the entire TEF-1 ORF (residues 2−426) functioned as a weak transactivator, whereas a chimera from which the TEF-1 DBD has been deleted (Δ55−121) was a much stronger activator. Analogous observations have previously been made using GAL4−AP-2, GAL4−E2−2 and CTF-1−Sp1 chimeras where the function of a strong activating domain was artefactually masked, apparently by the presence of two functional DBDs (Mermod *et al.*, 1989; Henthorn *et al.*, 1990; Williams and Tjian, 1991). The molecular basis of this effect is at present unknown. In the case of TEF-1, however, the low transactivation by the chimera containing both the TEF-1 and GAL4 DBDs apparently does not result from its inability to bind to the GAL4 site, nor from its inability to interact with the cognate TIFs, as evidenced by the fact that this chimera interferes with the activity of the endogenous TEF-1 as efficiently as the full length wild-type TEF-1.

Further mutagenesis of the GAL4−TEF-1Δ55−121 chimera, which stimulated transcription up to 60-fold, indicated that deletion of the C-terminal 34 amino acids or the STY-rich region resulted in almost a complete loss of transactivation. However, further analysis of these mutated chimeric proteins indicated that they did not form stable complexes in EMSA with oligonucleotides containing a GAL4 binding site. Thus, from this transactivation assay it cannot be definitively concluded that the STY-rich and C-terminal regions are part of the activating domain *per se*. Nevertheless, the fact that chimeras lacking these two regions did not interfere with the activity of endogenous TEF-1, does provide strong evidence indicating that these regions are required for interaction with a limiting intermediary factor(s) and hence, may also play important roles in transactivation.

The chimera GAL4−TEF-1(205−426), containing the two regions described above, did not function as a transcriptional activator. The minimal requirement for activation by TEF-1 was amino acids 205−426 together with at least the C-terminal portion of the proline-rich region, and/or the N-terminal acidic and 121−143 regions. Deletion of either of these regions leads to a 2- to 3-fold reduction in transactivation, but deletion of both regions completely abolishes activation. These observations clearly indicate that, while these regions may be partially redundant, they contribute to transcriptional activation by TEF-1. However, chimeras containing either of these two regions alone did not function as transcriptional activators. Taken together, the above results indicate that, in HeLa cells, at least three regions contribute to transactivation by TEF-1, but when fused individually to a heterologous DBD these regions do not homosynergize, nor do they heterosynergize with the upstream elements of the tk promoter present in the UAS_G-tk-CAT reporter. At present, however, we cannot exclude that, in other cell types or in other promoter contexts, these TEF-1 regions would function as autonomous activating domains.

Three of the regions of TEF-1 described above have distinctive amino acid contents analogous to those found in many other activators. Proline-rich regions or regions with high STY content have been shown to contribute to activation in transactivators such as CTF/NF-1, OTF(Oct)-2 and AP-2 (Mermod *et al.*, 1989; Gerster *et al.*, 1990; Tanaka and Herr, 1990; Williams and Tjian, 1991), or GHF-1, Bicoid, myogenin, ITF-1 and ITF-2 (Theill *et al.*, 1989; Struhl *et al.*, 1989; Schwartz *et al.*, 1992; Seipel *et al.*, 1992). In the case of AP-2, for example, deletion of the proline-rich region reduces activation in the wild-type AP-2 context, but unlike the proline-rich region from TEF-1, the AP-2 proline-rich region functions as an activating domain when fused to the GAL4 DBD (Williams and Tjian, 1991). Similarly the TEF-1 N-terminal region has a net negative charge and contains a high concentration of serines which are potential sites for phosphorylation. Although the role of acidic residues and phosphorylation in transactivation by many other factors has previously been established (Cress and Triezenberg, 1991; Hunter and Karin, 1992; Jackson, 1992, and references therein), the functional importance of the proline or STY residues *per se* has not been determined. In this context it should also be noted that the STY-rich region is also rich in potential phosphorylation sites, notably for casein kinase II (amino acid 310), or protein kinase C (amino acid 323) which have been shown to be regulators of transcription factor activity (De Groot and Sassone-Corsi, 1992; Lin *et al.*, 1992; Voit *et al.*, 1992; and references therein). Further studies will be required to elucidate the potential role of phosphorylation in the regulation of TEF-1 activity.

We have previously suggested (Xiao *et al.*, 1991) that the dominant negative phenotype of ectopically expressed recombinant TEF-1 in HeLa cells was due to a transcriptional interference effect resulting from the titration of a limiting intermediary factor by an excess of the TEF-1 activating domain. The *Drosophila* Krüppel protein is another transactivator which has recently been shown to have a dominant negative phenotype at low concentrations (Sauer and Jäckle, 1991), suggesting that the Krüppel AF is also mediated by a limiting TIF. In the case of TEF-1, the results of the present study show that GAL4−TEF-1 chimeras that do not function as transactivators are also unable to interfere with the activity of endogenous HeLa TEF-1, while chimeras which function as transactivators are also active in the interference assay. These conclusions are further supported by the results obtained with deletions in wild-type TEF-1. In this context also, deletion of the C-terminal region (mutant Δ402C) completely abolished self-interference. This C-terminal region has the potential to form a zinc finger, and it has been suggested previously that such a potential is important for the transactivation function of the adenovirus E1A gene product (Lillie and Green, 1989; Webster and Ricciardi, 1991, and references therein). However, in TEF-1, mutation of the cysteine or histidine residues had no effect on self-interference indicating that the potential to form a zinc finger was apparently not required for interaction with the cognate TIFs. Further mutational analysis of this region will be required to determine precisely which residues are involved in its function. Thus, taken together, the present

results strongly support the hypothesis that the stimulation of transcription by TEF-1 is mediated by a highly limiting TIF(s) which can be titrated by an excess of a functional TEF-1 activating domain.

As discussed above, there is a general correlation between transactivation and transcriptional interference by a given GAL4−TEF-1 chimera. A notable exception is the chimera containing the entire TEF-1 ORF which was a weak transactivator, but interfered with endogenous TEF-1 as efficiently as wild-type TEF-1, and more efficiently than the strongest activator GAL4−TEF-1Δ55−121. This result suggests that, in addition to the TEA/ATTS DBD, the Δ55−121 deletion may also have removed additional sequences involved in transactivation. Transactivation and interference results similar to those of GAL4−TEF-1Δ55−121 were obtained with another chimera (GAL4−TEF-1Δ46−100; our unpublished results). The results obtained with both of the above chimeras suggest that the sequences contributing to transactivation may be intimately associated with the DBD itself as in lambda repressor, glucocorticoid receptor, MyoD, myogenin and HAP-1 (Bushman *et al.*, 1989; Schena *et al.*, 1989; Weintraub *et al.*, 1991; Davis and Weintraub, 1992; Schwartz *et al.*, 1992; Turcotte and Guarente, 1992). Although there is presently no way to evaluate the transactivation potential of the entire TEF-1 ORF, due to the artificially low transactivation obtained with the GAL4−TEF-1(2−426) chimera, the above interference results strongly suggest that maximal transcriptional activation may also require the entire TEF-1 ORF. Thus, taken together the results of the present study imply that the fully conserved three dimensional structure of TEF-1 may be required for maximal interaction with the cognate TIFs leading to transactivation and transcriptional interference.

The TEA/ATTS domain is not the sole determinant of the DNA binding specificity of TEF-1 or scalloped

The results of the present study provide the first experimental evidence that the conserved TEA/ATTS domain is a novel conserved class of DBD. This short conserved domain alone is necessary and sufficient for binding to the GT-IIC and Sph enhansons, and none of the mutations in this domain had a differential effect on binding to these enhansons. Mutation of conserved residues in the first α-helical and third α-helix/β-sheet region have a strongly detrimental effect on specific DNA binding. However, mutation of the second predicted α-helix/β-sheet region had no effect on specific DNA binding. As the mutations consisted of the replacement of conserved amino acids with prolines, this result suggests that the α-helicity of this second region is not essential for its function, and that it may in fact adopt a β-sheet structure. Alternatively, this region may not be involved in DNA binding *per se*, but may be required to mediate interaction with other factors, analogous to the interaction of VP16 with helix 1 of the Oct(OTF)-1 homeodomain (Lai *et al.*, 1992; Pommerantz *et al.*, 1992). As discussed, the transcription interference results obtained with GAL4−TEF-1 chimeras containing deletions Δ55−121 or Δ46−100 also suggest that sequences within the DBD may play a role in transactivation.

The present results indicate that deletion of the C-terminal 23 amino acids and/or the STY-rich region has a negative effect on DNA binding mediated by the TEF-1 and GAL4 DBDs. In this respect it should be noted that mutation of the cysteine and histidine residues in the C-terminal zinc finger-like structure did not have a detrimental effect on DNA binding (our unpublished data). As the C-terminal and STY-rich regions are not part of the minimum DNA binding domain, their function may be to antagonize the negative effect of another region of TEF-1. A similar situation has recently been described in ets-1 and ets-2. In this case a domain adjacent to the ets/DBD has an inhibitory effect on DNA binding possibly as a result of its ability to change the structure of the ets domain itself (Wasylyk *et al.*, 1992). As the STY-rich region contains many potential phosphorylation sites, a process known to regulate the DNA binding of several transcription factors, phosphorylation of this region may modulate its function and allow TEF-1 DNA binding to be regulated by signal transduction pathways. Further studies will be required to elucidate how different domains of TEF-1 modulate DNA binding.

The TEA/ATTS domain of the *Drosophila sd* protein differs in only one amino acid from that of TEF-1, yet for *sd* no binding *in vitro* to either the GT-IIC or Sph enhansons was detected. Such a result is reminiscent of those obtained with members of the superfamily of helix−turn−helix/homeo(POU)domain proteins, where for example a single amino acid change in recognition helix 3 of the *paired* gene homeodomain is necessary and sufficient to convert the binding specificity of Paired to that of Bicoid or Fushi tarazu (Treisman *et al.*, 1989). Similarly, the binding specificity of the estrogen and glucocorticoid receptors is determined by two or three amino acids located in the first zinc finger (Danielsen *et al.*, 1989; Mader *et al.*, 1989). The present results suggest that, as the *in vitro* DNA binding specificities of TEF-1 and *sd* are different, the first α-helical region of the TEA/ATTS domain may be a recognition helix functionally equivalent to the third helix of the homeodomain. In agreement with this idea, conversion of the *sd* TEA/ATTS sequence to that of TEF-1 resulted in binding of the *sd* protein to the GT-IIC enhanson. However, the binding of this mutated *sd* protein to the GT-IIC enhanson was significantly weaker than that of TEF-1, and no binding to the Sph enhansons could be detected. Nevertheless, when the TEF-1 TEA/ATTS domain sequence was present in the context of a C-terminally truncated *sd* protein (*sd* Δ170C), binding to both the GT-IIC and Sph enhansons could be detected, while the truncated wild-type *sd* protein recognized neither of these enhansons. These results indicate that although the serine to alanine mutation in the TEA/ATTS domain did allow a change in DNA binding specificity, the effect of this change was modulated by the C-terminal region of *sd*.

Strikingly, in the converse experiment the conversion of the TEF-1 TEA/ATTS domain to that of *sd* had no effect on the *in vitro* binding of TEF-1 to the GT-IIC or Sph enhansons. Similar results were obtained when the TEF-1 A48 was changed to R as in the TEC-1 protein (our unpublished results). Thus, in agreement with the results obtained with the *sd* protein, the above results indicate that although the TEA/ATTS domain is a determinant of sequence-specific DNA binding, other regions of both the TEF-1 and *sd* proteins must also be involved. Further experiments will be required to determine exactly which other regions of the TEF-1 and *sd* proteins contribute to the binding specificity, and how they modulate recognition by the TEA/ATTS domain.

Materials and methods

Expression vectors and reporter plasmids

The expression vectors pXJ40, pXJ40-TEF-1A, pXJ40-GAL4(1−147), pXJ40-GAL4(1−147)−TEF-1(2−426) and pXJ40-GAL4(1−147)−TEF-1 (167−426), and the reporter plasmids pXJ40-LacZ, 2GTIIC(R)-tk-CAT, pBLCAT8+ and UAS_G-tk-CAT were as previously described (Webster *et al.*, 1988; Xiao *et al.*, 1991). The pXJ40-*sd* expression vector was constructed by PCR amplifying amino acids 1−440 of *sd* (generously given by S.Campbell and A.Chovnick) with oligonucleotide primers containing *Hin*dIII and *Not*I restriction sites. The resulting fragment was subcloned between the *Hin*dIII and *Not*I sites in pXJ40. Deletions and point mutations in wild-type TEF-1, GAL4−TEF-1 chimeras or *sd* were introduced by site-directed mutagenesis using single stranded DNA generated from pXJ40, -TEF-1A, -GAL4(1−147)−TEF-1(2−426), -GAL4(1−147)−TEF-1 Δ55−121 or -*sd* respectively, as previously described (Xiao *et al.*, 1991). In all C-terminal deletions the last amino acid of TEF-1 (426) was conserved along with the natural stop codon. To create TEF-1 Δ205C, amino acids 1−204 of TEF-1 were PCR amplified from pXJ40-TEF-1A and subcloned between the *Eco*RI and *Bgl*II sites of pXJ40. Similarly amino acids 2−45, 143−204, 205−426, 306−426 and 328−426 of TEF-1 were PCR amplified from pXJ40-TEF-1A and subcloned into the *Kpn*I and *Bgl*II sites of pXJ40-GAL4(1−147) (Xiao *et al.*, 1991). Amino acids 28−60, 28−85 and 28−104 were amplified by PCR and cloned in-frame between the *Eco*RI and *Bam*HI sites of pGEX2T (Smith and Johnson, 1988). The structures of all of the mutations and deletions were verified by DNA sequencing. The DNA sequences of the mutants created by PCR were determined completely, except in the cases of the longest TEF-1 construct (amino acids 205−426) or *sd*. In all cases, however, similar results were obtained from several independent clones.

Transfections and CAT assays

Transfection of HeLa cells was performed by calcium phosphate precipitation as previously described (Fromental *et al.*, 1988; Xiao *et al.*, 1991). Cells were transfected with 1 μg of reporter plasmid, 1 μg of the pXJ40-LacZ internal reference plasmid, 16 μg of pBluescript as carrier and the indicated quantities of the expression vectors. Cell extracts were made 48 h post-transfection. Transfection efficiency was first standardized by β-galactosidase assays and the appropriate amounts of cell extracts were then tested by CAT assays as previously described (Webster *et al.*, 1988; Xiao *et al.*, 1991). CAT assays were quantified following thin layer chromatography by scintillation counting or by Phosphorimage analysis.

Western blotting

Cytoplasmic and nuclear extracts from transiently transfected HeLa cells were prepared according to Hoppe-Seyeler *et al.* (1991). Equivalent amounts of protein were then separated by denaturing SDS−PAGE (Laemmli, 1970), and transferred to nitrocellulose. TEF-1 was detected using the anti-P1 serum described by Xiao *et al.* (1991), which recognizes the first 11 amino acids of TEF-1. GAL4−TEF-1 chimeras were detected using a mixture of two monoclonal antibodies, 2GV3 and 3GV2, directed against the GAL4 DBD as described by White *et al.* (1992). The blots were subsequently developed using an ECL kit (Amersham).

Transcription and translation in vitro

The appropriate pXJ40 vectors were linearized with *Bgl*II for TEF-1 or *Kpn*I for *sd*, downstream of the stop codons. 2 μg of linearized plasmid were then transcribed with T7 RNA polymerase as previously described (Hwang and Curthoys, 1991) in the presence of 500 μM sodium $m^7G(5')ppp(5')G$ (Pharmacia). 3 μg of each RNA were then translated using a nuclease-treated rabbit reticulocyte lysate in the presence of [^{35}S]methionine and the translation products were detected by autoradiography following SDS−PAGE and treatment with EN^{3}HANCE (Du Pont).

Electrophoretic mobility shift assays and preparation of bacterial extracts

Equivalent amounts of protein from the *in vitro* translations or *E.coli* extracts were mixed with 50 000 c.p.m. of the ^{32}P-5′-end-labelled double stranded oligonucleotides and 5 ng (500 ng when bacterial extracts were used) of poly(dI)·poly(dC) as nonspecific competitor DNA in a 25 μl reaction volume. The complexes were separated on 6% polyacrylamide gels in 0.5×TBE. The gels were then dried and subjected to autoradiography. The sequences of the wild-type (OGT2-50) GT-IIC probe, the dimeric GT-IIC enhanson probe (OGT2-56), and the wild-type (OSph-0) and mutated (OSph-5) Sph probes were as previously described (Davidson *et al.*, 1988; Xiao *et al.*, 1991), while in the mutated GT-IIC oligonucleotide the GT-IIC enhanson sequence, 5′-GTGGAATGT-3′, was changed to 5′-GTACGATGT-3′. The oligonucleotides containing the wild-type or mutated GAL4 binding sites were as previously described (White *et al.*, 1992).

Extracts from *E.coli* harbouring the pGEX2T-TEF-1 constructs were prepared by growing the bacteria to an OD_{600} of 0.6 and inducing with 1 mM IPTG for 2 h. The bacteria were then harvested, resuspended in 20 mM Tris−HCl pH 7.9, 10% (v/v) glycerol, 0.2 mM EDTA, 1 mM dithiothreitol, 0.1% NP40 and 200 mM KCl, and sonicated. The insoluble material was removed by centrifugation and the presence of the induced proteins in the soluble fraction was verified by SDS−PAGE and staining with Coomassie blue.

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