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Transcriptional activation by CTF proteins is mediated by a bipartite low-proline domain

(NFI gene/CTF family of proteins/proline-rich domain)

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ABSTRACT **Members of the CCAAT-binding transcription factor (CTF) family of proteins stimulate the initiation of adenovirus DNA replication and act as transcriptional activators. To investigate the mechanisms underlying CTF-mediated transactivation patterns, we expressed several natural CTF variants in *Saccharomyces cerevisiae* and determined their transactivating activities in enzymatic assays. CTF7, which lacks the entire proline-rich region previously thought to mediate transcriptional activation by CTF proteins, enhances transcription to a greater degree than full-length CTF1, which contains the putative activation domain. CTF2, which contains a partially deleted proline-rich activation region, does not stimulate transcription at all. These findings indicate that the proline-rich region of CTF proteins is not essential for transcriptional activation in yeast. Our studies also suggest a bipartite two-domain structure of CTF-type transcriptional activation domains.**

Transcriptional activation by many DNA sequence-specific regulatory factors is thought to be mediated via protein domains rich in either acidic side chains, glutamine residues, or proline residues (1). Recent data suggest that these domains are recognized by a class of polypeptides, termed TATA box binding protein-associated factors, or TAFs, which are part of the multisubunit protein complex, TFIID (2, 3), that plays a pivotal role in controlling RNA polymerase II-dependent transcription.

The prototype of the proline-rich class of activators is a family of closely related DNA-binding proteins termed CCAAT-binding transcription factors, or CTF proteins (4, 5). CTF proteins arise from a single cellular gene by translation of alternately spliced mRNAs (see Fig. 1*A*) (4–6). They form homo- and heterodimers that interact with the consensus sequence 5′-TGG(N)$_6$GCCAA-3′ (4, 7–9). This sequence often occurs in regions of cellular and viral genomes with putative enhancer or promoter functions (10–13). CTF proteins have been shown both to stimulate the initiation of adenovirus type 2 and 5 DNA replication *in vitro* and *in vivo* (14–16) and to activate transcription from a number of cellular and viral promoters. Other proteins in this class of cellular DNA-binding proteins participating in the initiation of transcription and DNA replication are the octamer-binding factor OCT-1 (17, 18), the yeast mating factor MCM-1 (19), and the general regulatory factor RAP1/GRF-1 (20, 21).

While DNA-binding and dimerization domains of CTF proteins have been studied in considerable detail, the structure of the transactivation domain remains unknown. In the present study we determined transactivation activities of various natural splice variants of CTF proteins in *Saccharomyces cerevisiae*. It will be shown that they display widely different activities depending on their particular exon arrangement. Thus it is not just proline richness alone that is the key to transcriptional activation; rather, these regions appear to be composed of spacially separated domains, thereby permitting naturally derived splice variants arising from a single gene to display distinctive transactivation activities.

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MATERIALS AND METHODS

Isolation and Characterization of CTF7 cDNA. The isolation of genomic porcine CTF clones from an EMBL3A library and the screening of a commercially available λgt11 porcine liver cDNA library (Clontech; PL 1001 B) with a *Pst* I/*Sac* I fragment coding for 141 amino acids from exon 2 has been described (4, 22). The CTF7 clone with a functional ATG as the first codon was cloned into *Sma* I-linearized pGEM-4Z (Promega) downstream of the SP6 promoter. Capped transcripts were synthesized *in vitro* from this expression clone in the presence of 500 mM m^{7}GpppG. RNA was treated with RNase-free DNase, phenolized, purified on Sephadex G-50 columns, and precipitated with 1 vol of isopropyl alcohol. *In vitro* translations were carried out in rabbit reticulocyte lysates (Promega) at 30°C for 2 hr. Protein mixtures were analyzed for quality and size on SDS/polyacrylamide gels and for DNA-binding activities on native polyacrylamide gels. One to 3 μl out of 50 μl of a reticulocyte extract was incubated in buffer A [25 mM Hepes/KOH (pH 7.8), 150 mM KCl, 5 mM EDTA, 1 mM dithiothreitol, and 10% (vol/vol) glycerol] containing 1 μg of poly(dI-dC) and 30 fmol of the unlabeled DNA fragment L1/2 for 20 min at 20°C and subsequently loaded onto a 12% native polyacrylamide gel. L1/2 is a 33-bp oligonucleotide containing one specific CTF binding site as described (4). In DNA competition assays, L1/2 and a 10- or 25-fold molar excess of competitor DNA was incubated for 10 min at 0°C. The sequence of L1/2 is 5′-GGGCTTTTGGCACTGTGCCAACTGTGTTGTGA-3′.

Plasmid Construction. Plasmids were constructed by standard techniques and used for transformation of *Escherichia coli* JM83 (Stratagene). Plasmid pLR1Δ1, which does not contain the upstream activator site (UAS) region, has been described (22). Reporter plasmids pLR-CTF3x, pLR-CTF6x, pLR-lexA, and pLR-lexA2x were constructed from pLR1Δ1 by inserting a synthetic consensus CTF or LexA operator into the *Xho* I site of the plasmid, 167 nucleotides upstream from the primary transcription start site of the *GAL1–lacZ* fusion gene.

pSH2-1 and pEG202 (gifts of R. Brent, Massachusetts General Hospital, Boston), which constitutively direct the synthesis of LexA hybrids in *S. cerevisiae*, were used to generate LexA-(1–87)–CTF and LexA-(1–202)–CTF fusions. Cloning of CTF cDNAs into the polylinker region downstream of the coding sequence of the bacterial repressor LexA-(1–87) resulted in the expression vectors pSH-CTF1, pSH-CTF1-(1–7), pSH-CTF1-(5–9), pSH-CTF1-(8–11),

Abbreviations: CTF, CCAAT-binding transcription factor; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside.

pSH-CTF1-(9–11), pSH-CTF2, pSH-CTF2-(8–10), pSH-CTF7, and pSH-CTF7-(5–11). The same CTF fragments were cloned into expression vector pEG202, resulting in LexA-(1–202)–CTF fusions. The positive control LexA–GAL4 has been described (23). The negative control LexA–bicoid was a gift from R. Brent.

For constitutive expression of CTF cDNAs, the vector pAAH5 (24) was linearized with *Hin*dIII, and the ends were made blunt by treatment with the Klenow fragment of DNA polymerase I. Insertion of CTF cDNAs resulted in the expression vectors pAA-CTF1, pAA-CTF2, and pAA-CTF7.

Cell Culture, Transformation, and Enzymatic Assays. Bacterial strains were grown in Luria–Bertani medium containing the appropriate amounts of antibiotics. Wild-type strains of *S. cerevisiae* were grown on yeast extract/peptone/dextrose medium or, when they contained plasmids, on minimal medium (25).

Growth selection was done on leucine-deficient plates. Yeast cells were made competent by treatment with lithium acetate (26). For assays of transcriptional activity, cells were transformed both with expression and with reporter plasmids. Indicator plates contained 40 mg of 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) per liter and 70 mM potassium phosphate buffer adjusted to pH 7.0. The amount of β-galactosidase in liquid cultures of yeast transformants was determined as described (27, 28). Single colonies from yeast transformants were inoculated in 5 ml of medium, grown overnight at 30°C, diluted 1:10 the next day, and grown to an optical density of about 0.8 at 600 nm. Between 0.02 and 0.1 ml of culture was added to Z buffer (60 mM $Na_2HPO_4 \cdot 7H_2O$, 40 mM $NaH_2PO_4 \cdot H_2O$, 10 mM KCl, 1 mM $MgSO_4$ at pH 7.0) up to a total volume of 1.0 ml. Each sample subsequently received 0.05 μl of 0.01% SDS and three drops of chloroform prior to mixing. Units of β-galactosidase activity obtained with *o*-nitrophenyl β-galactoside (4 mg/ml in 0.1 KH_2PO_4) were normalized for the optical density at 600 nm of the culture as described. All assays were repeated five times using fresh and independent transformants. Reproducibility typically was ±10% among five transformants.

Antibodies and Western Blotting. Samples were subjected to electrophoresis, transferred to nitrocellulose, and blotted in the presence of PBS, 20% (vol/vol) fetal calf serum, and 3% (wt/vol) dry milk for 1 hr at 37°C (blocking solution). Subsequently, the filter was incubated with anti-LexA antibody in PBS, 0.05% Tween 20, and 20% fetal calf serum for 3 hr at room temperature, rinsed, and washed twice with PBS/0.05% Tween 20. After incubation for 1 hr with the secondary antibody to alkaline phosphatase, the nitrocellulose was washed in 10 mM Tris·HCl, pH 8.0/150 mM NaCl/0.05% Tween 20 and developed with nitroblue tetrazolium (50 mg/ml in 70% dimethylformamide) and 5-bromo-4-chloro-3-indolyl phosphate (50 mg/ml in dimethylformamide) reagent (Promega).

RESULTS AND DISCUSSION

In the present study we have analyzed transcriptional activation patterns of three CTF proteins, CTF1, CTF2, and CTF7 (Fig. 1). CTF1, the largest of the three proteins, is 507 amino acids long and represents the product of all 11 exons of the NF1 gene. It thus contains the entire proline-rich C-terminal region of this protein family. CTF2 lacks exon 9, and a change in reading frame results in termination within exon 10. These changes remove most of the proline-rich region present in CTF1 and generate a protein of only 438 amino acids. CTF1 and CTF2 have been described (4, 5). CTF7 represents an isolate that was obtained as a cDNA clone by screening a porcine cDNA library with a 423-bp *Pst* I/*Sac* I fragment covering parts of the second exon of CTF. This cDNA clone has a coding sequence of 1063 bp, corresponding to only 351 amino acids. CTF7 lacks exons 7–9, which are present in full-length CTF1, and thus is 156 amino acids shorter than CTF1 (Fig. 1*B*). Since deletion of exons 7–9 does not alter the reading frame for exons 10 and 11, CTF7 has the same amino acid sequence as CTF1 except for deletion of most of the proline-rich region thought to be involved in transcriptional activation. CTF7, harboring the whole N-terminal region, binds as a dimer to the CTF recognition sequence $TGG(N)_6GCCAA$ and thus behaves indistinguishably from the other products of the CTF gene with respect to these parameters (data not shown).

Transcriptional activation of CTF1, CTF2, and CTF7 was studied in an *S. cerevisiae* system originally devised by Brent and Ptashne (23). In this system, a LexA fusion protein with a putative transcriptional activator, when tethered to DNA sites with the appropriate DNA sequences, activates expression of a target gene (e.g., *lacZ* or *LEU2*) (Fig. 2). The level of expression of the target gene is a measure of the ability of a given protein to act as a transcriptional activator.

The reporter plasmids used in the present study contained either a CTF recognition sequence or a LexA binding site in the upstream region of the *GAL1–lacZ* fusion. Binding of an active CTF or LexA–CTF fusion protein to the respective binding sites on the reporter plasmid stimulates the expression of the GAL1–LacZ fusion protein, which can be quantitated by a β-galactosidase assay.

Expression vectors were introduced into yeast strain MGDL4a or EGY48 that contained the reporter constructs described in *Materials and Methods*. Expression plasmids carried the *S. cerevisiae* LEU2 or HIS3 selection marker and an alcohol dehydrogenase (*ADH*) promoter in front of either a gene encoding a CTF protein or a gene encoding the N-terminal DNA-binding domains of the bacterial repressor LexA [LexA-(1–87) or LexA-(1–202)] fused to different CTF sequences.

Gel retardation assays were used to determine functionality of CTF and LexA–CTF proteins. Specific binding was demonstrated by heterologous and homologous competition (data not shown). The fact that the LexA fusion proteins bind indistinguishably from authentic CTF proteins is an important control ruling out the possibility of LexA fusion proteins displaying reduced and/or different DNA-binding activities as compared to protein LexA itself (29). In addition to the functional gel retention assays, the proper expression of the LexA fusions was demonstrated in Western blotting experiments with anti-LexA antisera (data not shown). CTF proteins and their LexA fusion derivatives are thus being expressed with similar efficiency in *S. cerevisiae*.

The ability of CTF- and LexA–CTF proteins to activate transcription on the reporter construct was monitored *in vivo* by conversion of X-Gal (Fig. 3). Expression of CTF7 and CTF1 resulted in a significant activation of GAL1–LacZ expression; CTF1 was less active than CTF7. Significant activity was also detected with a CTF7 deletion construct lacking the N-terminal parts of CTF1 represented by exons 1–4 (Fig. 1*A*). CTF1-(1–7), CTF1-(8–11), and CTF1-(9–11) also show weak activity. No visible expression was observed from clone CTF2, the N-terminal deletion construct CTF2-(8–10), and a CTF1 deletion clone harboring only exons 1–5 or exons 5–9. Identical results were obtained with LexA-(1–202) fusions. The presence of the LexA-derived dimerization domain thus does not influence our results.

All LexA fusions were also screened for complementing leucine auxotrophy mediated by their respective transactivating abilities and shown to behave similarly as those expressing β-galactosidase. In these selections, we tested LexA-(1–87) fusions with CTF1 exons 1–7 (domain I; see below), exons 8–11 and exons 9–11 (domain II), and exons 8 and 10 from CTF2. The CTF1-derived exons displayed

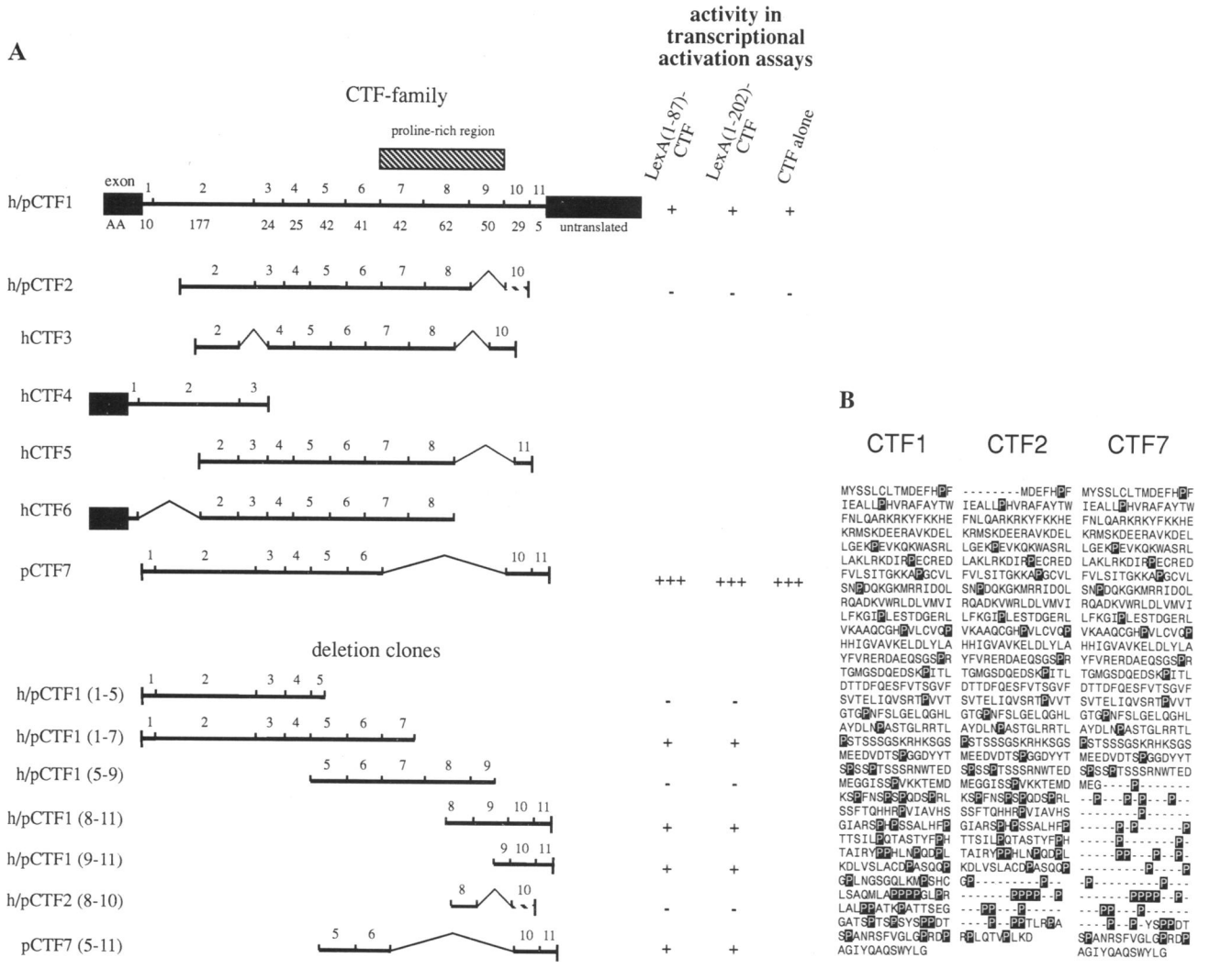

FIG. 1. Schematic representation of the CTF cDNA family generated by alternative splicing from a single CTF gene. (*A*) All clones were isolated from HeLa or porcine liver cDNA libraries as described (4, 5). In this figure they are designated as either pCTFn (p for porcine) or hCTFn (h for human). Human and porcine clones are used interchangeably since they share an overall homology of >97% and do not display any amino acid changes in the regions relevant to this work. The proline-rich region is indicated by a hatched box above the CTF1 full-length clone. Plus or minus signs indicate the respective transcriptional activation activities of the various constructs. A single plus sign represents approximately one-fifth of the activity of the fully active clone pCTF7. (*B*) Sequence comparison of CTF1, CTF2, and CTF7. The entire amino acid sequences of the three proteins are represented in one-letter code, demonstrating the differences in the proline-rich domain. Proline residues are indicated by inverse lettering. The spliced exons in CTF2 (exon 9) and CTF7 (exons 7–9) are shown as dashes. Splicing of CTF2 results in a frame shift and a translational stop in the 10th exon, whereas splicing of CTF7 does not alter exons 10 and 11. The deletion of exon 1 in clone CTF2 does not influence DNA binding (4, 9).

weekly positive activation, whereas the C-terminal exons 8 and 10 from CTF2 were totally inactive (Fig. 1*A*).

As positive controls, *S. cerevisiae* clones transformed with a LexA–GAL4 fusion protein formed deeply blue colonies on appropriate indicator plates (Fig. 3). Negative controls included a LexA–bicoid fusion (29), the reporter plasmids alone in the absence of LexA or LexA–CTF expression plasmids, and transformants with reporter plasmids lacking LexA or CTF-binding sites (data not shown). These latter controls demonstrate that *S. cerevisiae* does not contain endogenous proteins that are able to recognize LexA or CTF-binding sites and that stimulate expression from the reporter gene.

Extracts from most transformants were also assayed with *o*-nitrophenyl β-galactoside as a substrate (Table 1). Columns 2 and 3 show the transcriptional activity of the reporter plasmids with LexA binding sites in the presence of LexA–CTF fusions. The positive control, LexA–GAL4, had 500 units of activity while the negative control, LexA–bicoid, showed little or no β-galactosidase expression (<2 units, background).

Transcriptional activity of the LexA–CTF1 fusion was 40 units, whereas no activity (2 units) was detected for LexA–CTF2 and the deletion mutant LexA–CTF1-(5–9). In contrast, the LexA–CTF7 fusion expressed 180 units of β-galactosidase, about 4.5 times more than the CTF1 fusion and at least 200 times more than LexA–CTF2. As controls, transformants containing only reporter plasmids with binding sites for LexA or CTF displayed only background activity.

The LexA–CTF fusion clones LexA–CTF1, LexA–CTF2, and LexA–CTF7, which contain the DNA-binding domains of both LexA and CTF, are active in the presence not only of a reporter driven by LexA-binding sites but also of a reporter driven by CTF-binding sites (Table 1). The activities are similar to those determined with LexA-binding sites. Fusions between LexA–CTF1-(5–9) and LexA–CTF7-(5–11), which lack a functional CTF DNA-binding domain, located in the N-terminal 264 amino acids (4, 9), showed no detectable transcriptional activity in this assay. Likewise, the LexA–GAL4 fusion protein did not stimulate GAL1–LacZ transcription from reporter plasmids with CTF-binding sites.

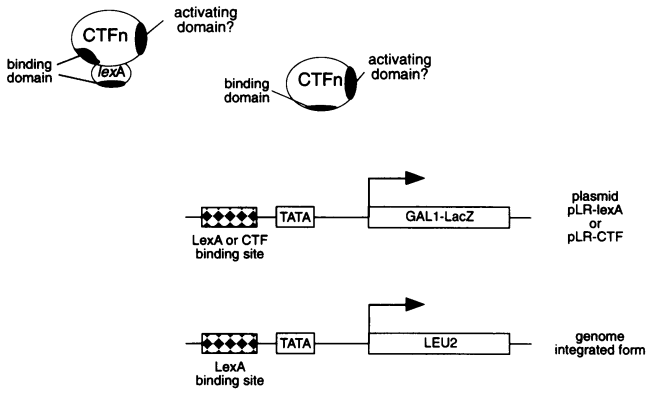

FIG. 2. Assay system for CTF-dependent transcriptional activation. To determine the transcriptional activity of the different CTF proteins we used a β-galactosidase assay and a LEU2-based growth selection. The *lacZ* and *LEU2* reporter genes are only expressed in this assay if the protein binding in the promoter region stimulates the transcription machinery. The reporter plasmids used in these studies were supplied with three or six CTF-binding sites and one, two, or six LexA-binding sites to determine specific transcriptional activation via CTF or LexA–CTF proteins. The product of the *lacZ* gene (β-galactosidase) confers a distinctive blue color to yeast colonies on appropriate X-gal indicator plates or a clear yellow color, detectable in a quantitative assay, using a chromogenic substrate, *o*-nitrophenyl β-galactoside, instead of X-Gal. In the absence of transcriptional activation mediated via the CTF or LexA–CTF proteins, the colonies remain white, and no color is detected in the quantitative assay.

Strains that express native CTF proteins lacking the fused LexA domain display similar activities when expressed in the presence of the *lacZ* reporter driven from CTF-binding sites. CTF7 (180 units) enhanced gene expression of the *lacZ* reporter gene as efficiently as the LexA–CTF7 fusion. Transcriptional activity of the full-length clone CTF1 was 42 units. In this assay, CTF2 led to a detectable but very weak stimulation of β-galactosidase expression (4 units). Negative controls lacking the CTF expression plasmids or lacking CTF-binding sites on the reporter plasmids did not activate expression of the *lacZ* gene.

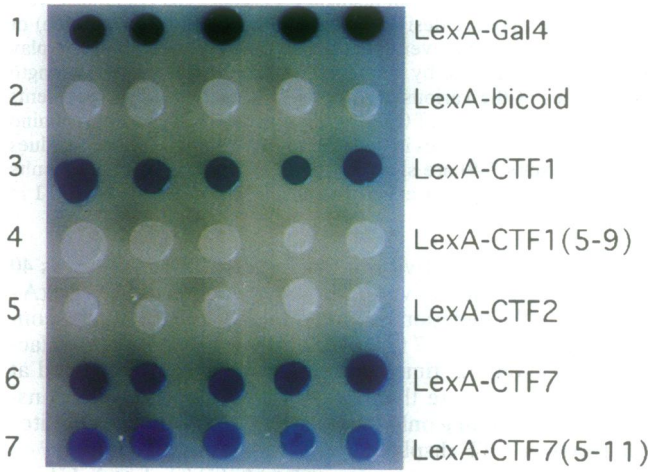

FIG. 3. Results of qualitative β-galactosidase assays. To assay for transcriptional activation, competent yeast cells were transformed with expression and with reporter plasmids. Indicator plates contained X-Gal. We used five independent transformants harboring a reporter plasmid with two LexA-binding sites. The positive control, LexA–GAL4, in the top row turned deeply blue, and the negative control, LexA–bicoid, in the second row remained white as expected. Row 3, LexA–CTF1; row 6, LexA–CTF7; and row 7, LexA–CTF7-(5–11) show blue color, indicating the stimulatory effect of these fusion proteins on *lacZ* gene expression. LexA–CTF1-(5–9) in row 4 and LexA–CTF2 in row 5 remained white, indicating the absence of transactivation activity.

We thus conclude that three alternate splice products of the CTF gene display a wide spectrum of transcriptional activation activities ranging from none (CTF2) to intermediate (CTF1) and strong (CTF7) activator activity. The latter approaches an activity not far from that of the prototype activator protein LexA–GAL4. This result was totally unexpected since the observed activation activities are, at least in part, inversely related to the presence of the proline-rich transactivation domain. Removal of a part of this region, represented by exon 9, resulted in a complete loss of transcriptional activation, whereas deletion of the entire proline-rich activation domain, represented by exons 7–9, resulted in significant stimulation of its transcriptional activation activity in *S. cerevisiae*.

How can these observations be understood? One possible model postulates the presence of a bipartite transactivation domain (Fig. 4), with domain I, residing within exons 4–6, and domain II, located within exons 10 and 11. Expressed separately, they display only weak stimulatory activation (Fig. 4). We suggest that these two domains become functionally fully active only in close contact with each other. In CTF1 they are separated by the proline-rich polypeptide domain of 156 amino acids (exons 7–9; Fig. 1*B*) that permits only a limited interaction of the two domains allowing an intermediate activation activity. This limited activity could be mediated by the exon 9-driven proline-rich polypeptide domain, presumably representing a protein domain of high flexibility. CTF2 lacks not only this flexible exon 9 linker but also domain II, which is lost due to a change in the reading frame of CTF2. It thus should be inactive, which is indeed the case. In contrast, CTF7 not only carries both domains I and II but also lacks the entire linker region that separates them in CTF1. The two activation domains thereby come into close proximity and interact to display the full transactivation activity.

At present the exact role of the bridging region represented by exons 7–9 can only be speculated upon. The proline-rich region derived from exon 9 may be needed as a flexible arm to modulate the interactions between the two regulatory domains in CTF1. Exons 7 and 8 may contain a binding site for a repressor protein, which may interfere with the inter-

Table 1. Results of quantitative β-galactosidase assays

	Binding sites				
	LexA		CTF		
Construct	1×	2×	3×	6×	None
LexA–GAL4	500	510	<2	<2	<2
LexA–bicoid	<2	<2	<2	<2	<2
LexA–CTF1	38	40	40	42	<2
LexA–CTF1-(5–9)	<2	<2	<2	<2	<2
LexA–CTF2	2	2	2	2	<2
LexA–CTF7	175	180	182	182	<2
LexA–CTF7-(5–11)	35	37	<2	<2	<2
CTF1	ND	ND	42	45	<2
CTF2	ND	ND	4	4	<2
CTF7	ND	ND	180	183	<2

For quantitation of the transcriptional activity of CTF proteins, both expression and reporter plasmids were transformed into competent yeast cells. The values given are β-galactosidase units (μmol of *o*-nitrophenyl β-galactoside per min). Transformants harboring reporter plasmids with one (1×), two (2×), or no (None) LexA-binding sites were used to quantify transcriptional activity of LexA-(1–87)–CTF fusions. Transformants harboring reporter plasmids with three (3×), six (6×), or no (None) CTF-binding sites were used to quantify transcriptional activity of CTF proteins and LexA–CTF fusions. ND, not determined.

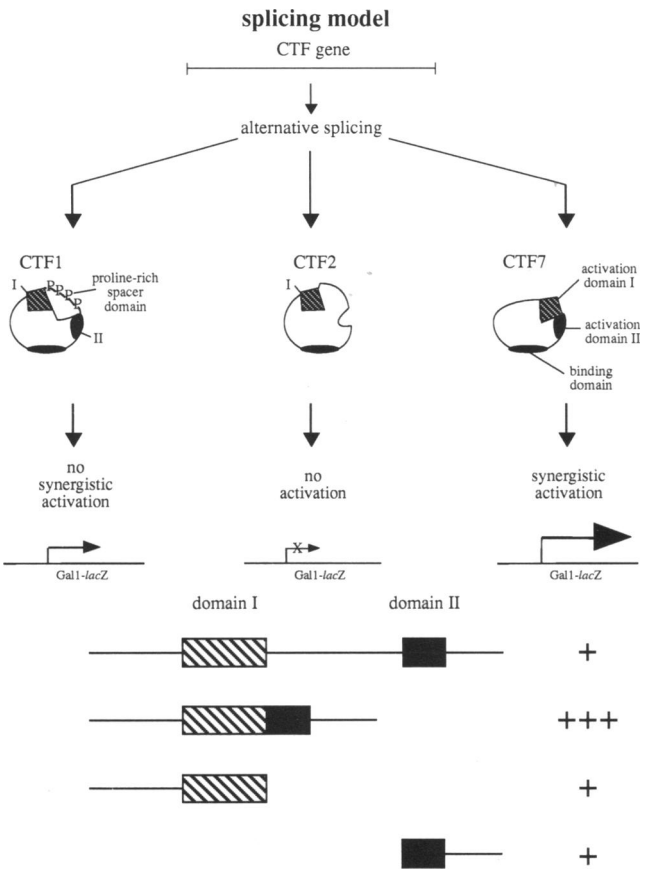

FIG. 4. Model for transcriptional activation in CTF proteins. CTF1 contains two domains (I and II), which are required to modulate transcriptional activation separated by a proline-rich spacer region. The full-length protein displays weak transactivational activity. CTF2 lacking part of the proline-rich domain and the entire domain II is not able to activate transcription from a reporter gene. CTF7, a product lacking the entire proline spacer, has domain I and II in close proximity to each other. This constellation enables CTF7 to enhance gene expression by permitting interaction of its two putative transactivation domains.

action of domains I and II in CTF1. An activity binding to this region has been described by Dusserre and Mermod (30).

The question remains whether our observations made in the *S. cerevisiae* system are relevant for mammalian systems. There is good reason to believe not only that the basic transcription machinery is highly conserved between yeast and man (reviewed in ref. 31) but also that the yeast system can even be used to functionally clone the corresponding protein from mammalian systems involved in various aspects of transcriptional control (cf. refs. 32 and 33). Recently it has even been shown that yeast transcription factor TFIID can support both basal and activated transcription in *in vitro* transcription reactions reconstituted with the respective human counterparts (34). These data, however, include experiments studying transcriptional activation mediated through protein GAL4–VP16 (i.e., through acidic and not through glutamine-rich or proline-rich domains). The possibility thus remains that the specific aspect of CTF-activated transcription is different in *S. cerevisiae* from mammalian cells although this is unlikely. However, this particular question can be answered experimentally as well as the bipartite activator model itself.

Our study suggests (*i*) that different CTF proteins derived as natural splice variants can display widely different transactivation activities in *S. cerevisiae*, (*ii*) that the proline-rich transactivation domain is not active as such but rather appears to display a bipartite structure, and (*iii*) that as a means for regulating gene expression a broad range of transactivating activities can be realized in a protein family simply by creating alternative splice variants.

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