CTF5—a new transcriptional activator of the NFI/CTF family

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ABSTRACT

NFI/CTF is a family of polypeptides involved in stimulating the initiation of adenovirus DNA replication and the activation of transcription driven by RNA polymerase II. Several naturally occurring NFI/CTF variants display distinctive transactivation activities in vivo. To define more precisely the role of the NFI/CTF family in regulating gene expression, we cloned the splice variant CTF5, analyzed transcriptional activation patterns in a yeast transcription assay, and compared it with other CTF proteins. CTF5, which lacks exons 9 and 10 including a CTD-like motif essential for transcriptional activation by full-length CTF1, enhances transcription to a greater extent than CTF1. In addition, CTF5 is even more active than CTF7, which lacks exons 7–9. These findings indicate that CTF proteins formed by differential splicing display a much broader range of transcriptional activities as observed previously.

INTRODUCTION

The accurate and regulated transcription of genes by RNA polymerase II is achieved by the combined action of three classes of protein factors. One class comprises the general transcription factors that are required for the ordered assembly of an active preinitiation complex (reviewed in 1). Regulation of the basal machinery involves the participation of various sequence-specific regulatory factors. These are usually characterized by the existence of separate DNA binding and activation domains (2,3). The third class of protein factors function as coactivators mediating interactions between the general and sequence-specific transcription factors (reviewed in 4,5).

Stimulating factors can be classified according to the amino acid compositions of their activation domains (3). They are rich in either acidic side chains, glutamine residues, proline residues or isoleucine residues (3,6).

Although the precise mechanisms by which different types of activation domains stimulate transcription are not known, it has been shown that the mechanisms of action for acidic and proline-rich activation domains are fundamentally similar in eukaryotic organisms from yeasts to humans (2,7,8). For example, the acidic activation domain of yeast transcription factor Gal4 is able to stimulate transcription in Drosophila (9), tobacco (10), hamster (11) and human cells (12). On the other hand, acidic activation domains of several mammalian transcriptional activators such as the herpes virus protein VP16 (13), the Jun oncoprotein (14), and the p65 subunit of NF-κB (15) activate transcription in Saccharomyces cerevisiae.

The proline-rich activation domain of the human activator CTF is also functional in Drosophila and yeast (16,8). In particular, CTF1-enhanced TFIIIB recruitment was observed in both human and yeast systems (17). We and others previously showed that transcriptional activation by CTF1 depends on a sequence motif strongly related to the C-terminal domain (CTD) of RNA polymerase II (18–20). Furthermore, our analysis of the transactivating activities of several natural CTF variants in S.cerevisiae demonstrated that the proline-rich region of CTF proteins alone is not essential for stimulating transcription (21). To gain an understanding of the mechanisms underlying CTF-mediated transactivating activities we have extended our previous studies on the NFI/CTF family by characterizing a new member, the splice variant CTF5. Our results show that CTF5, which lacks exons 9 and 10, activates transcription in S.cerevisiae to a greater degree than all CTF splice forms analyzed so far.

Since CTF5 lacks part of the proline-rich domain and does not contain the CTD-related motif of full-length CTF1 this suggests that CTF proteins affect gene expression through the generation of alternate splice variants containing functionally different transactivation domains.

MATERIALS AND METHODS

Media and strains

Media were prepared according to standard methods (22).

Plasmid construction was performed using Escherichia coli strain DH10B (Gibco-µBLR Life Technologies), and bacteria were grown in LB medium containing the appropriate amounts of antibiotics.

In vivo studies were carried out in the yeast strain S.cerevisiae EGY48: MATa, trp1, ura3, his3, LEU::pLexAop6-LEU2 (23). Yeast cells were grown on yeast YEPD medium, strains
containing plasmids were grown on minimal medium as described elsewhere (24).

Plasmids

Reporter plasmids pLR-CTF6x (21) and pSH18-34 (23) were constructed from pLR1Δ1 by inserting synthetic consensus CTF or LexA operators. Plasmid pLR1Δ1, which does not contain the upstream activator site (UAS) region, has been described (25).

Expression vectors pSH2-1 and pEG202 (gifts of R. Brent, Massachusetts General Hospital, Boston) constitutively direct the synthesis of LexA-(1–87) and LexA-(1–202) hybrids in S. cerevisiae.

Expression plasmids pSH/-pEG- CTF1, pSH/-pEG- CTF2 and pSH/-pEG- CTF7 have been described previously (21).

The control plasmids pSH-Gal4 (26) and pEG-hicoid (27) were a gift from the laboratory of Dr R. Brent. They were used to generate the positive control, LexA-(1–87)-Gal4, and the negative control, LexA-(1–202)-bicoid.

Construction of expression plasmid CTF5

Phages containing both full-length and partial CTF5 cDNA clones were isolated from a HeLa cDNA library (Clontech). A particular insert with which this work had originally started was subcloned in pUC12 (pUC-CTF5) and found to start with amino acid residue 85 within exon 2 (28). To complete the N-terminus of the CTF5 sequence, an XbaI/Kle/BglII deletion was created in pGEM4Z-CTF1 and substituted by an EcoRI/Kle/BglII fragment of pUC-CTF5 (EMBL accession number X92857). This fragment includes the CTF5-characteristic exon junction from exon 8 to 11.

The reconstituted plasmid pGEM4Z-CTF5 was used as a donor for an EcoRI/EcoRI-CTF5 fragment, which was cloned into the EcoRI-linearized S. cerevisiae expression vectors pSH2-1 and pEG202 to yield the CTF5 expression plasmids pSH-CTF5 and pEG-CTF5. These were used to generate the LexA-(1–87)-CTF5 and LexA-(1–202)-CTF5 fusion proteins.

Coupled in vitro transcription/translation

The commercially available TNT™SP6-coupled Reticulocyte Lysate System from Promega was used according to the manufacturer’s instructions. Full-length CTF1 and the splice variants CTF2, CTF5, CTF7 were translated directly from pGEM4Z-CTF plasmids by addition to the TNT Lysate and by incubation in a 50 µl reaction volume for 2 h at 30°C. The newly synthesized proteins were analyzed by SDS–PAGE and autoradiography.

Yeast transformation and enzymatic assays

Yeast cells were made competent for transformation by treatment with lithium acetate (29). For assays of transcriptional activity cells were transformed both with expression and reporter plasmids. Growth selection was performed on leucine-deficient media and by conversion of X-Gal.

RESULTS

CTF5—a new member of the NFI/CTF family

In the present study we have compared the transcriptional activation activity of CTF5, a new member of the NFI/CTF family, with CTF proteins CTF1, CTF2 and CTF7 characterized previously (21). Full-length CTF1 represents the product of the 70 kb NFI/CTF gene, consisting of 11 exons. It thus contains an N-terminal DNA binding and dimerization domain as well as the ‘prototype’ proline-rich transactivation domain located at the C-terminus (Fig. 1A). The derivative CTF2 lacks exon 9 as a result of alternative splicing. Removal of this exon creates a frame shift and termination within exon 10. CTF7 lacks exon 7, 8 and 9. The splice process however does not alter the reading frame for exons 10 and 11. CTF1, CTF2 and CTF7 have been described previously (32,33,21).

CTF5 represents a cDNA clone which was isolated by screening a HeLa cDNA library with a 423 bp PstI–SacI fragment covering parts of the second exon of NFI/CTF. CTF5 lacks exons 9 and 10 and thus contains neither the entire proline-rich region nor the CTD-related motif, shown to be essential for transcriptional activator function of CTF1 (18–20).

To prove the coding capacity of the full-length protein products of the various NFI/CTF cDNAs, the appropriate proteins were expressed by coupled in vitro transcription and translation (Fig. 1B).

CTF-dependent ‘transcription assay’ in S. cerevisiae

A CTF5 cDNA was cloned into the yeast expression vectors pSH2-1 and pEG202 to obtain appropriate LexA-(1–87)-CTF5 and LexA-(1–202)-CTF5 fusion proteins. Analogous LexA–CTF expression plasmids of all four CTF proteins were introduced into S. cerevisiae strain EGY48 containing different reporter constructs. Functional expression of LexA–CTF proteins in transformed EGY48 yeast cells has been described previously (21). To monitor CTF-dependent transcriptional activation we used a chromosomal Leu2 growth selection and a reporter plasmid based β-galactosidase assay (Fig. 2).

Saccharomyces cerevisiae strain EGY48 harbouring an integrated LexA indicator gene under exclusive control of six LexA binding sites (23). The β-galactosidase assay was performed by cotransformation of expression and reporter plasmids carrying either eight LexA binding sites (pSH18-34) or six CTF recognition sequences (pLR-CTF6x) upstream of the Gα1–lacZ fusion (23,21). In both assay systems reporter genes are expressed only if the protein binding to the promoter region is able to act as a transcriptional activator. The ability of the LexA–CTF proteins to stimulate transcription of the reporter constructs was therefore monitored as growth on leucine-deficient media and by conversion of X-Gal.
Figure 1. (A) Comparison of full-length CTF1 with the natural splice variants CTF2, CTF5 and CTF7. CTF1 is 507 amino acids long and consists of 11 exons which are distributed along the NFI/CTF gene. It contains the N-terminal DNA binding and dimerization domain indicated by a hatched box. The C-terminal proline-rich transactivation domain (= squared box) of CTF1 includes the CTD-related motif SPTSPSYSP around the junction of exon 9 and 10. CTF2 lacks exon 9; a concomitant change in reading frame results in a translational stop within exon 10 generating a protein of only 439 amino acids. CTF5, which is derived by alternative splicing of exons 9 and 10 from the NFI/CTF gene, encodes a protein of 428 amino acids. In CTF7 the exons 7–9 are spliced, it is thus 156 amino acids shorter than CTF1. All spliced CTF forms have the N-terminal DNA binding and dimerization domain whereas none of them harbours the complete CTD-like motif. (B) In vitro transcription and translation of the various CTF proteins yielded polypeptides of the expected size.

Figure 2. Schematic representation of the expression and reporter constructs used for CTF-driven ‘transcription assays’. Four members of the NFI/CTF family were fused to the DNA binding domain of LexA (amino acids 1–87) or to the DNA binding and dimerization domain of LexA (amino acids 1–202). These expression constructs were assayed by introduction into yeast cells containing different reporter constructs. *Saccharomyces cerevisiae* reporter strain EGY48 harbours a Leu2 gene carrying LexA binding sites in its promoter region. The reporter plasmids for β-galactosidase assays contained LexA binding sites (pSH18-34) or CTF binding sites (pLR-CTF6x) in the upstream region of the Gal1–lacZ fusion.

**CTF5 activates transcription stronger than all other CTF splice variants**

Due to their respective transactivating activities, the LexA fusions of CTF1, CTF5 and CTF7 were capable of complementing leucine auxotrophy (data not shown). Expression of LexA–CTF2, however, failed to activate transcription. The ability of LexA–CTF transformed yeast cells to grow on leucine-deficient media was independent of the presence of the LexA-derived dimerization domain. Cells grew to an equal extent with either LexA–(1–87) or LexA–(1–202) constructs. As expected, the positive control LexA–Gal4 strongly stimulated growth on selection plates, whereas the negative control LexA-bicoid did not.

The transcriptional activation activity of LexA–CTF proteins was also determined by enzymatic conversion of X-Gal as a result of CTF-stimulated expression of β-galactosidase (Fig. 3). LexA fusions of CTF1, CTF5 and CTF7 stimulated Gal1–lacZ transcription as efficient as they were shown to activate expression of the Leu2 reporter gene. LexA–CTF2 fusions again were inactive. The positive control turned blue and the negative control remained white, as expected.
Results of β-galactosidase assays. To determine transcriptional activation of CTF proteins competent yeast cells were cotransformed with expression and reporter plasmids. Binding of transcriptionally active LexA-(1–87)–CTF fusion protein in the promoter region via LexA binding sites results in stimulation of the lacZ gene expression. On X-gal indicator plates respective transformants turned blue. For quantitation ONPG was used as substrate. Relative transcriptional activation rates of CTF proteins we normalized with respect to the strongest activator, CTF5, setting its activation level to 100%. For comparison the results of the negative and positive control obtained with the LexA-bicoid and LexA–GAL4 fusions are shown.

In addition to analyzing transcription of the lacZ indicator gene via LexA binding sites, the LexA–CTF fusion proteins were also tested for expressing β-galactosidase via CTF binding sites (data not shown). The fact that the observed transactivating abilities are indistinguishable from each other demonstrates that the LexA-fusion part does not influence functional expression of CTF proteins.

The results of β-galactosidase assays with the LexA-(1–87) and the LexA-(1–202) constructs showed some differences. Judging from the blue colour intensities it appears that the presence of the LexA dimerization domain leads to a less intense phenotype as compared to transformants carrying only the LexA binding domain. Nevertheless, apart from the LexA-fusion part, the results of qualitative β-galactosidase assay demonstrate that CTF5 stimulates transcription to a higher extent than CTF1.

To determine more precisely the transcriptional activity of CTF5 we quantified the levels of Gal1–lacZ expression in a liquid β-galactosidase assay with o-nitrophenyl β-galactoside (ONPG) as substrate (Fig. 3). For this purpose *S. cerevisiae* strain EGY48 was transformed with LexA-(1–87) fusions of CTF1, CTF2, CTF5 and CTF7 together with reporter plasmids pSH18-34 or pLR-CTF6x. If the maximal level of transcriptional activity detected for LexA–CTF5 was set to 100%, only background activity (0.7%) was observed for LexA–CTF2. The LexA–CTF7 fusion then expressed 45%, and the LexA–CTF1 fusion to 20% of the β-galactosidase activity level observed for the CTF5 fusion. In comparison, the positive control with the yeast transcription factor GAL4-derived fusion construct, LexA–GAL4, yielded a 610% stimulation, whereas the commonly accepted negative control construct, LexA-bicoid, was not distinguishable from background activities. Furthermore, transformants harbouring reporter plasmids with binding sites for LexA or CTF, but no expression plasmid, showed background activity as well. In addition, the quantified β-galactosidase activity levels of the LexA–CTF fusions gave the same results when the reporter was driven by LexA binding sites or CTF binding sites. Transcriptional activation activities thus are independent of the nature of the DNA binding domain that is being used (data not shown).

We conclude that different CTF proteins derived as differential splice variants display a broad spectrum of transactivating activities ranging from none (CTF2), over weak (CTF1) to intermediate (CTF7) and strong (CTF5) enhancement rates.

**DISCUSSION**

Alternative splicing from the single NFI/CTF gene generates several protein products that show striking diversity of transactivation activities. In this work, we have demonstrated that the newly characterized CTF5 variant is the strongest activator of the NFI/CTF family.

This is somehow surprising, since CTF5 lacks part of the proline-rich domain as well as the CTD-related motif. Both regions have been reported to be important for CTF1 transcriptional activation activity. Analysis of various CTF1 deletions mutants had revealed that a transcriptionally active domain is contained within the proline-rich C-terminal 100 amino acids of CTF1 (16). Therefore the proline-rich region in CTF1 was assumed to be a
novel and distinct activation domain motif for transcription factors (16,3). In support of this conclusion, certain amino acid substitutions located in the amino acid residues 470–479, displayed decreased transcriptional activation rates (18).

In CTF5, which lacks exons 9 and 10, part of the proline-rich (aa 423–502) domain and the entire CTD-related (aa 470–479) motif are missing. Thus, transcriptional activation by CTF5 cannot be due to the presence of the CTD-related motif. The functional significance of the residual proline-rich domain remains to be determined.

It has been suggested that the removal of part of the proline-rich domains can result in an increase of transcriptional activation activity (21,34). CTF7, lacking exons 7–9, lacks most of the proline-rich domain but still contains part of the CTD-related motif. Compared with CTF1 this splice variant exhibits a significantly higher transcriptional activation activity and therefore other transcriptional activation mechanisms have been proposed (21). In addition, differential splicing of another CTF-related gene, the NFI-X gene, has been shown to yield three splice variants, NFI/X1, NFI/X2 and NFI/X3 (34,35). NFI/X2, which in comparison to the other NFI/X variants lacks part of the proline-rich activation domain, is the most potent transcriptional activator (34).

Taken together, the remarkable transcriptional activation activity of CTF5 is not due to the presence of the CTD-related motif and presumably does not depend on the proline-rich domain. Instead it must be assumed that this activity is mediated by different sequence motifs. Requirement for the observed activation activity through the residual proline-rich region remains to be examined, but since CTF7 lacks this particular region and still stimulates transcription stronger than CTF1, it cannot be responsible for the observed transcriptional activation activities. Therefore, at present the sequence motif requirements underlying the diverse splice variants of NFI/CTF are not known.

One possible explanation for the strong transactivating activity of CTF5 despite the absence of known activating sequence motifs may come from the recently reported regulatory effects of the NFI/X proteins (35). NFI/X2, which lacks part of the proline-rich domain, activates transcription to a similar extent as NFI/CTF1. NFI/X1 shows no activation activity in human cells (34). Furthermore, the recently described variant, NFI/X3, differs from NFI/X1 by an additional insertion of a 49 amino acid sequence, which causes a frame-shift of subsequent C-terminal sequences. It shows significant transcriptional repressor function. Deletion analysis identified the regulatory region of NFI/X between amino acids 130 and 280. All NFI/X proteins contain this 'X-repressor domain', although they exhibit different regulatory effects on transcription. It is assumed, therefore, that other regions in NFI/X proteins with positive regulatory capabilities may modulate the action of this negative regulatory sequence. Differences in transactivating activities of NFI/CTF variants could be explained, therefore, by the variable presence of different repressor and activator functions as well.

Another explanation of the different transcriptional activating activities of NFI/CTF isoforms has been discussed in the context of the recently described HNF1 homeoprotein family (36). The isoforms HNF1-B and HNF1-C, which are generated by the differential use of polyadenylation sites as well as by alternative splicing, differ in their C-termini from the full-length mRNA derived isoform HNF1-A. Both shorter isoforms are stronger transactivators than HNF1-A. Their different transactivation potential is explained by: (i) the interaction of HNF1-B and/or HNF1-C with the same mediator protein as HNF1-A (with interactions occurring on a different domain of this mediator protein), or (ii) the interaction of HNF1-B and/or HNF1-C with a different mediator protein which can induce higher transcriptional activation (36).

Presently little is known about the interactors of CTF-depend-ent transcription. Recent studies suggest TFIIB (17), TBP (19,37) and TAFII55 (38) as targets for CTF1 during RNA polymerase II-driven transcription. Possibly, the alternative splicing of the NFI/CTF gene generates proteins with different binding specificities for potential interactors, especially coactivators and/or repressors, resulting in the observed broad range of transactivating activities.

Apart from the participation of several mediators responsible for the different transactivation potentials of NFI/CTF isoforms other mechanisms such as modulated affinity for certain promoter contexts or different influence on chromatin structure cannot be excluded.

The question remains whether data from S.cerevisiae are relevant for mammalian systems. This notion is supported by several observations. The ongoing characterization of the basal transcription machinery of S.cerevisiae continues to reveal an unexpectedly close relationship with that of higher eukaryotes. Furthermore, the discovery of TAFs in S.cerevisiae underlines that the activation mechanisms for transcription seem to be very similar as well (39–41).

One recent example of the applicability of results gained in S.cerevisiae arises from studies on transcription factor Fos. There, it has been shown that the exchange of amino acid residues within a transcriptional activation domain has the same influence on transcription in the mammalian system (42).

Therefore we assume that our analysis of CTF-stimulated transcription made in the S.cerevisiae system is also relevant for mammalian systems.

Our results suggest (i) that several CTF proteins derived as natural splice variants activate transcription to different extents and (ii) that the potential to obtain factors with different activities from the same gene adds a further dimension to the mechanisms existing for the modulation of gene expression.

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