

## Two Distinct Domains in Staf To Selectively Activate Small Nuclear RNA-Type and mRNA Promoters

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**Staf is a transcriptional activator of prime importance for enhanced transcription of small nuclear (snRNA) and snRNA-type genes transcribed by RNA polymerases II and III (Pol II and III). In addition to this activity, it also possesses the capacity to stimulate expression from an RNA polymerase II mRNA promoter. This promiscuous activator thus provides a useful model system for studying the mechanism by which one single transcription factor can activate a large variety of promoters. Here, we report the use of *in vivo* assays to identify the Staf activation domains involved in promoter selectivity. Analysis of Staf mutants reveals the existence of two physically and functionally distinct regions, outside of the DNA binding domain, responsible for mediating selective transcriptional activation. While a 93-amino-acid domain, with the striking presence of four repeated units, is specialized for transcriptional activation of an mRNA promoter, a segment of only 18 amino acids, with a critical Leu-213 residue, acts specifically on Pol II and Pol III snRNA and snRNA-type promoters. In addition, this study disclosed the fundamental importance of invariant leucine and aspartic acid residues located in each repeat unit of the mRNA activation domain. Staf is therefore the first transcriptional activator described so far to harbor two physically and functionally distinct activator domains. This finding suggests that the same activator can contact different, specialized transcription complexes formed on different types of basal promoters through promoter-specific transactivation pathways.**

Transcriptional activation in eukaryotes is achieved by regulatory proteins recruiting the transcriptional machinery and chromatin remodeling factors to the promoter. Transcriptional activators have been shown to present a modular organization consisting of distinct domains for sequence-specific DNA binding and transcriptional activation through interaction with other proteins (for a review, see reference 34). DNA binding domains of many transactivators fall into readily identifiable classes. Instead, domains responsible for transcriptional activity may not possess such a well-defined organization. While no obvious sequence similarity has been detected among the activation domains of diverse activator proteins, they nevertheless often carry a distinctive amino acid composition. For example, activation domains particularly rich either in prolines, glutamines, acidic amino acids, or hydroxylated amino acids have been described (for reviews, see references 4, 21, 27, and 51). Despite the identification of targets for numerous activation domains and elucidation of the functional importance of particular residues, the structural basis for the capacity of an activation domain to stimulate transcription remains poorly understood. However, recent structural studies suggest that the  $\alpha$ -helix may be a common structural motif in the binding of transactivation factors to transcriptional activation factors (22, 52).

Transcriptional activators were also described to stimulate transcription of RNA polymerases II (Pol II) and III (Pol III) small nuclear RNA (snRNA) genes. In vertebrates, these genes contain an essential proximal sequence element (PSE) located at approximately the position  $-59$  upstream of the transcription start site. The Pol III-dependent promoters possess addi-

tionally a TATA box located at position  $-30$ , which acts as a major determinant for RNA Pol III specificity (24, 26; for a review, see reference 15). A number of other short transcription units, such as the 7SK, Y, MRP, selenocysteine tRNA (tRNA<sup>Sec</sup>), and H1 RNA genes, have similar basal promoter elements and can be classified as snRNA-type genes (3, 30; for a review, see reference 14). These will be here referred to as snRNA-type promoters. SNAPc, also called PTF, is a stable complex containing four protein subunits. It binds specifically to the PSE (13, 31, 37, 54, 55). Other studies have also implicated the TATA binding protein (TBP), TFIIC1, and TFIIB $\alpha$  in transcription of Pol III snRNA genes (25, 41, 48, 55).

Associated with the basal promoter, the snRNA and snRNA-type genes contain a distal sequence element (DSE) around position  $-220$ , which plays a major role in transcription efficiency. The DSE contains an octamer motif which binds the transcriptional activator Oct-1 (42; for reviews, see references 14 and 16). In addition to Oct-1, Sp1 has been shown in some instances to be involved in mediating the activation properties of the DSE (1, 20, 23, 47). Recently, we have demonstrated that the zinc finger protein Staf, originally identified as the transcriptional activator of the tRNA<sup>Sec</sup> gene (39), is also involved in transcriptional activation of snRNA and snRNA-type genes transcribed by RNA polymerases II and III (38). In the course of previous investigations, we observed that Staf also possesses the ability to stimulate CAT expression from a Pol II promoter (32, 39), therefore showing that it can activate a whole diversity of Pol II and Pol III promoters. At first sight, this result might be surprising in view of the fact that, although transcription of mRNA and Pol II snRNA genes is catalyzed by the same enzyme, the architectures of mRNA and snRNA transcription complexes have some dissimilarities (for reviews, see references 14 and 15). So far, the mechanism by which Staf can interact with these different Pol II and Pol III transcription complexes has remained unknown. In order to gain insight into

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it, the mapping and functional dissection of the Staf activation domain was undertaken. We report here that Staf contains in fact two distinct snRNA and mRNA promoter-specific activation domains, explaining why a single transcriptional activator can activate a variety of different promoters. This finding lays the foundation for further elucidation of the detailed, promoter-specific activation pathways.

## MATERIALS AND METHODS

**Effector constructs.** Staf-Krox-20  $\Delta$ 473 to 600 (SK $\Delta$ 473-600), the Staf-Krox-20 3'-end-truncated derivative, was obtained by double digestion of pBRN3-Staf-Krox-20 (39) with *EcoRV* (cleavage at position 1443 of the Staf cDNA sequence) and *NorI* (cleavage in the polylinker of pBRN3) and recircularization of the plasmid after filling-in of the *NorI* 5' extremity. Staf-Krox-20  $\Delta$ 6-248 (SK $\Delta$ 6-248), the Staf-Krox-20 5'-end-truncated derivative, was obtained by digestion of pBRN3-Staf-Krox-20 with *NcoI* (cleaved at positions 138 and 771 of the Staf cDNA sequence) and recircularization of the filled-in ends of the shortened plasmid. Internal deletions  $\Delta$ 6-77,  $\Delta$ 6-102,  $\Delta$ 6-130,  $\Delta$ 6-206,  $\Delta$ 6-220,  $\Delta$ 78-220,  $\Delta$ 157-220,  $\Delta$ 6-206+ $\Delta$ 225-242,  $\Delta$ 84-176,  $\Delta$ 244-262, and  $\Delta$ 212-220 in Staf-Krox-20  $\Delta$ 473-600, truncation  $\Delta$ 212-220 in Staf-Krox-20, and mutants PDTIS207-211AAAA, SIEG221-224AAAA, E214A+Q215A+K219A, L213A+Y216A, S211A, L213A, E214A, Q215A, Y216A, and K219A in Staf-Krox-20  $\Delta$ 473-600 were obtained by site-directed mutagenesis of the parental vector. S84-176K contains the two internal deletions,  $\Delta$ 6-78 and  $\Delta$ 177-262, obtained by site-directed mutagenesis of the parental vector.

S207-224K was obtained as follows. A polylinker created by annealing the oligonucleotides 5'-GATCTTCTAGAGCCGCCACCATGGCATCGATCCCGGGAAGACACACCAAGGATCCAGATCTGCG3' and 5'-CATGGCAGATCTGGATCCTTGGTGTGTCTTCCCGGGATCGATGCCATGGTGGCGGCTCTAGAA3' was inserted into pBRN3-Krox-20-DBD cleaved at the *BglII* and *NcoI* sites (39). This creates E83. A 64-bp *BbsI* fragment (containing Pro 207 to Gly 224 of Staf), obtained by PCR amplification using the 5' and 3' primers complementary to positions 646 to 663 and 679 to 700 of the Staf cDNA sequence, was inserted into the *BbsI*-cleaved E83 construct. The 5'-CTGAAGACACACCACCCGACACAATCAGCGCA and 3'-CTGAAGACTGTGGTCTCCTTCAATCGACACCTTTC primers incorporated the *BbsI* sites. The sequence of the N-terminal part of the S207-224K protein upstream of the Krox-20-DBD is MASIPGKTHHPDTISALEQYAAKVSIEGDGGSRSAM (in bold, the Staf sequence from residues 207 to 224).

Deletion mutations in pPac-Staf (39) were obtained as follows. *BamHI* sites were introduced by site-directed mutagenesis of pBRN3-Staf (39) at positions 156 and 1839 of the Staf cDNA sequence to create pBRN3-Staf-BB. Deletions  $\Delta$ 6-248,  $\Delta$ 473-600,  $\Delta$ 84-176,  $\Delta$ 212-220,  $\Delta$ 77-102,  $\Delta$ 77-130,  $\Delta$ 77-156,  $\Delta$ 77-220,  $\Delta$ 103-220,  $\Delta$ 131-220, and  $\Delta$ 157-220 and mutants D168A+G169A+T170A, L166A, D168A, and T170A were created by site-directed mutagenesis of pBRN3-Staf-BB. The *BamHI* fragments encoding the Staf mutants were excised from the mutated pBRN3-Staf-BB and introduced clockwise at the *BamHI* site into plasmid pPac to generate the series of pPac-Staf expression constructs for transfection into *Drosophila* SL2 cells.

**Reporter constructs.** p6E.tkCAT, AE.tkCAT, 3E.tRNA<sup>Sec</sup>, and 3E.U1 are described in references 53, 32, 39, and 38, respectively.

**Oocyte microinjections, nuclear localization, and DNA binding assays.** Capped mRNAs were synthesized in vitro by T3 RNA polymerase as described in Schuster et al. (39) and were injected (20 nl, 1 ng) into the cytoplasm of *Xenopus laevis* oocytes, 20 h before nuclear injection of 20 nl containing the reporter. The concentrations of the reporters were 300  $\mu$ g/ml for 6E.tkCAT and 50  $\mu$ g/ml for 3E.tRNA<sup>Sec</sup> and 3E.U1. The 3E.tRNA<sup>Sec</sup> and 3E.U1 reporters were injected in the presence of [ $\alpha$ -<sup>32</sup>P]GTP (800 Ci/mmol, 0.2  $\mu$ Ci/oocyte) and 5S RNA maxigene (5  $\mu$ g/ml) as an internal control for nuclear microinjection and RNA recovery. The 6E.tkCAT reporter was injected in the presence of the pCH110 vector (300  $\mu$ g/ml) as an internal control for nuclear injection. Incubation was for 16 h (3E.U1 and 6E.tkCAT reporters) or 3 h (3E.tRNA<sup>Sec</sup> reporter). Transcription of the reporter genes was analyzed as described previously (32, 39). The transcription efficiencies of the tRNA<sup>Sec</sup> and U1 RNA genes, relative to the 5S RNA maxigene expression, were quantitated with a Fuji Bioimage Analyzer BAS2000. For monitoring synthesis and nuclear localization of the effector proteins, oocytes microinjected with mRNAs were manually enucleated. Oocyte nuclei were homogenized in 8  $\mu$ l of the extraction buffer [50 mM Tris-HCl (pH 8.0), 50 mM KCl, 0.1 mM EDTA, 5 mM MgCl<sub>2</sub>, 25% glycerol, 1  $\mu$ l of bestatin per ml, 1  $\mu$ l of pepstatin A per ml, 1  $\mu$ l of *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane per ml, 1  $\mu$ l of 4-(2-aminoethyl)-benzenesulfonyl fluoride per ml] per nucleus. Homogenates were clarified by centrifugation at 13,000 rpm for 5 min. <sup>32</sup>P-labeled double-stranded oligonucleotides containing the E element were used for Staf-Krox-20 and Krox-20-DBD bandshift assays as described previously (6).

**Transfections, CAT assays, and Western analysis.** Schneider line 2 (SL2) *Drosophila* cells were transfected by the DOTAP liposomal transfection procedure (Boehringer). The cells received 500 ng of expression plasmid, 500 ng of reporter plasmid, and 200 ng of pACH110 (49).  $\beta$ -Galactosidase activity, normalization,

and chloramphenicol acetyl-transferase (CAT) assays were performed as described previously (39). Results were quantitated with a Fuji Bioimage Analyzer BAS2000. The data represent the averages of results from three to four transfections, each performed in duplicate. For Western blot analysis, extracts from *Drosophila*-transfected cells were prepared essentially as described previously (2). Proteins were transferred to nitrocellulose and visualized with a polyclonal serum raised against a C-terminal Staf peptide with the use of ECL reagents (Amersham).

## RESULTS

**Mapping of the Staf activation domain.** The Staf amino acid sequence can be divided into three regions (Fig. 1A): an N-terminal domain (amino acids 1 to 263), which we refer to as domain A; a central DNA binding domain B containing seven zinc fingers of the C<sub>2</sub>-H<sub>2</sub> type (amino acids 264 to 468); and the C-terminal domain C (amino acids 469 to 600). Truncated Staf proteins were constructed, in order to define the Staf domains which are needed for the activation of snRNA, snRNA-type, and TATA box-containing mRNA promoters. The transactivation capabilities provided by the wild-type and truncated proteins (Fig. 1A) was assayed on various promoters by microinjection into *X. laevis* oocytes. To eliminate the background provided by the endogenous Staf in this assay, we designed chimeric Staf-Krox-20 proteins with altered DNA binding specificities. The Staf zinc finger domain was replaced by the corresponding sequences of the Krox-20 zinc finger domain (K-DBD), as illustrated in Fig. 1A (5, 39). This results in chimeric Staf-Krox-20 molecules (Fig. 1A) with different DNA binding specificities but overall structures that are likely to be relatively unchanged from that of the wild-type protein. The abilities of the different Staf-Krox-20 chimeric proteins to activate snRNA and snRNA-type promoters were assayed with *Xenopus* U1b1 snRNA (Pol II) and tRNA<sup>Sec</sup> (Pol III) promoters. They contained, in place of the residing activator element, a multimerized version of the Krox-20 binding site E element (3E.U1 and 3E.tRNA<sup>Sec</sup>) (Fig. 1A). The reporter 6E.tkCAT (Fig. 1A), containing six E elements upstream of the *tk* promoter, was used for monitoring the transcriptional activation on TATA box-containing mRNA promoters (53).

The mRNAs of the various Staf-Krox-20 effectors were transcribed in vitro, capped, and injected separately into the oocyte cytoplasm (39). Twenty hours postinjection, the 6E.tkCAT, 3E.tRNA<sup>Sec</sup>, and 3E.U1 reporters were injected separately into oocyte nuclei. 3E.U1 and 3E.tRNA<sup>Sec</sup> were injected along with [ $\alpha$ -<sup>32</sup>P]GTP and a plasmid containing a 5S RNA maxigene as an internal standard. After a second incubation, the labeled RNAs were extracted and the transcription levels of the U1 snRNA and tRNA<sup>Sec</sup> genes, normalized relative to the 5S RNA maxigene expression, were used to measure the transactivation properties of the protein tested. For experiments with the mRNA promoter, a plasmid containing the  $\beta$ -galactosidase gene, not responding to Staf, served as an internal control and was coinjected with the 6E.tkCAT reporter into oocyte nuclei. After a second incubation, protein extracts were prepared and the normalized CAT activity was used as a measure of the transactivation properties. In the presence of Staf-Krox-20 (SK), the transcription levels of 6E.tkCAT, 3E.U1 and 3E.tRNA<sup>Sec</sup> were significantly enhanced (compare lanes 1 and 3 in Fig. 1B and lanes 1 and 2 and 5 and 6 in Fig. 1C). With SK $\Delta$ 473-600, a Staf-Krox-20 derivative lacking the C-terminal domain C, a comparable level of activation was obtained (compare lanes 3 and 4 in Fig. 1B and lanes 2 and 3 and 6 and 7 in Fig. 1C). This effect is not mediated by Krox-20-DBD, since transcription of the 6E.tkCAT, 3E.U1, and 3E.tRNA<sup>Sec</sup> reporters was not enhanced by the presence of this protein containing only the DNA-binding domain (Fig. 1B, lane 2) (38, 39). In contrast, removal

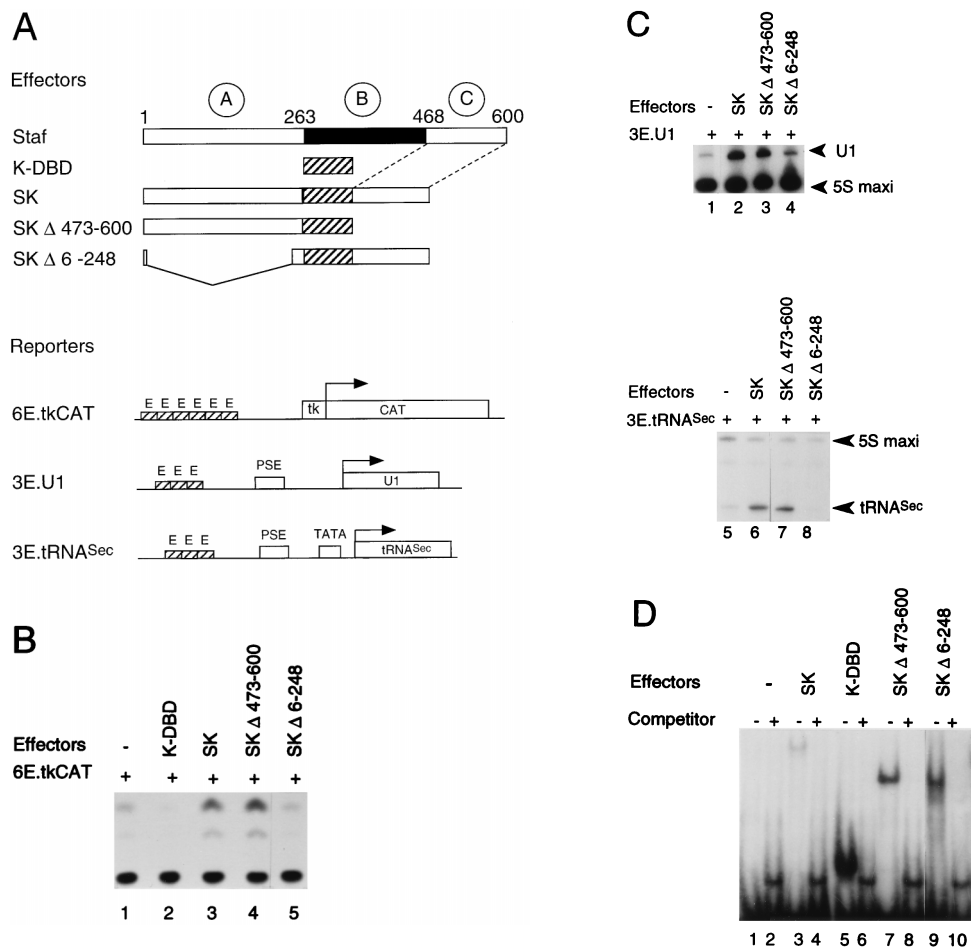


FIG. 1. The Staf-Krox-20 chimera requires the N terminus to activate transcription from tk.CAT, U1 snRNA, and tRNA<sup>Sec</sup> promoters. (A) Schematic diagrams depicting the effector mRNAs synthesized in vitro and the 6E.tkCAT, 3E.U1 and 3E.tRNA<sup>Sec</sup> reporter genes used in the *Xenopus* microinjection assay. The first lane shows the structure of Staf. The A, B, and C domains are indicated with their localizations. PSE and TATA correspond to the basal promoter elements of the U1 and tRNA<sup>Sec</sup> genes. 3E and 6E indicate the number of multimerized E binding sites of the Krox-20 protein. SK, chimeric Staf-Krox-20 molecules. (B) CAT assays showing transcriptional activation from the 6E.tkCAT promoter by the K-DBD, SK, SK $\Delta$ 473-600 and SK $\Delta$ 6-248 effectors using extracts from microinjected *Xenopus* oocytes. Lane 1, no effector was expressed. (C) Transcriptional activation from the Pol II 3E.U1 (lanes 1 to 4) and Pol III 3E.tRNA<sup>Sec</sup> (lanes 5 to 8) promoters by the SK, SK $\Delta$ 473-600 and SK $\Delta$ 6-248 effectors using microinjected *Xenopus* oocytes. Lanes 1 and 5, no effector was expressed. Positions of the U1, tRNA<sup>Sec</sup>, and 5S maxi used as an internal standard are indicated. (D) Effector expression assayed by gel retardation with nuclear extracts from microinjected *Xenopus* oocytes. The identity of the expressed effector is indicated above each lane. Lanes 1 and 2, no effector was expressed. Reactions in lanes 1, 3, 5, 7, and 9 (–) contained no competitor; lanes 2, 4, 6, 8, and 10 (+) contained a 100-fold molar excess of unlabeled E competitor DNA.

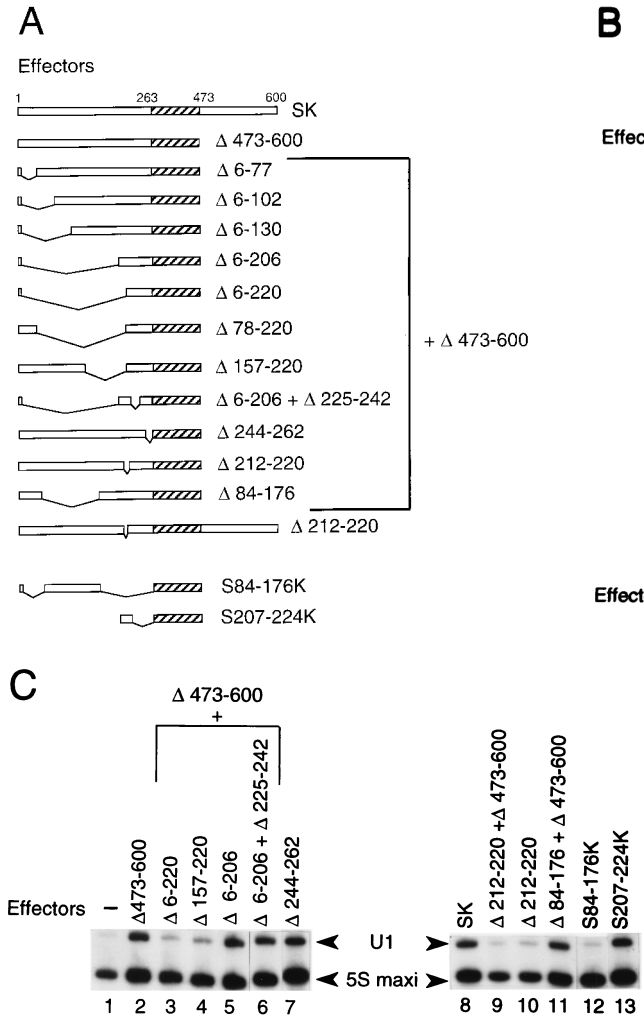
of the N-terminal amino acids 6 to 248 in SK $\Delta$ 6-248, resulted in the complete loss of enhanced transcriptional activity for the three reporters (compare lanes 3 and 5 in Fig. 1B and lanes 2 and 4 and 6 and 8 in Fig. 1C). Note that for lane 4, the normalized value for the U1 transcription level is very similar to that obtained in lane 1, taking into account that the intensity of the 5S maxigene internal control is higher in lane 4 than in lane 1.

The quantity, quality, and nuclear localization of the effector proteins were assayed by gel retardation assays with oocyte nuclear extracts and a <sup>32</sup>P-labeled oligonucleotide probe containing one Krox-20 E site (Fig. 1D). Competition experiments revealed the specificity of the shifted complexes since an excess of unlabeled E site competed efficiently with their formation (Fig. 1D). The relative mobilities of the different complexes indicated that full-length effector proteins were expressed in these microinjections. Quantitation of the shifted complexes showed that the relative binding efficiencies of the chimeric proteins SK $\Delta$ 473-600 and SK $\Delta$ 6-248 in the oocyte nuclear extracts are 10-fold higher than that of SK (Fig. 1D). However, these

binding efficiencies were not used to correct the activation levels, since titration experiments showed that the levels of the effector proteins are saturating in our assays; therefore, a 10-fold increase or decrease in the mRNA concentration of the effector will not change the level of transcriptional activation (data not shown).

Taken together, these results indicate that the N terminus of Staf (domain A) is necessary and sufficient for maximal transactivation of Pol II and Pol III snRNA and snRNA-type promoters and TATA box-containing mRNA promoters.

**The minimal activation domain for snRNA-type promoters is 18 amino acids long.** To define the minimal region of Staf sufficient for activation of snRNA and snRNA-type promoters by RNA polymerases II and III, the 263-amino-acid activation domain A was subdivided by creating a series of deletion mutants on Staf-Krox-20  $\Delta$ 473-600 (Fig. 2A). These fusion proteins were again tested for their ability to activate transcription of the 3E.tRNA<sup>Sec</sup> and 3E.U1 reporters in a microinjection assay. Internal deletions between amino acid 5 and amino acids



**FIG. 2.** Characterization of the minimal activation domain for Pol II and Pol III snRNA promoters. (A) Schematic structures of the various truncated and fused Staf-Krox-20 proteins. The Krox-20 DBD is hatched. (B) *Xenopus* microinjection assay of transcriptional activation from the 3E.tRNA<sup>Sec</sup> promoter by the effectors depicted in panel A. Identities of the effector proteins are indicated above each lane. Lanes 9, 10, and 19, assays without effector (-). Positions of the 5S maxigene and tRNA<sup>Sec</sup> RNAs are indicated. Autoradiographs corresponding to lanes 1 to 9, 10 to 15, 16 to 18, and 19 to 20 were from separate experiments. (C) *Xenopus* microinjection assay of transcriptional activation from the 3E.U1 promoter by the effectors depicted in panel A. Positions of the U1 and 5S maxi RNAs are indicated. Lane 1, assay without effector (-).

78 (Δ6-77), 103 (Δ6-102), 131 (Δ6-130), and 207 (Δ6-206) did not impair activation of the Pol III tRNA<sup>Sec</sup> and Pol II U1 promoters (Fig. 2B, compare lane 1 and lanes 2 to 5 for the tRNA<sup>Sec</sup> promoter; and lanes 2 and 5 for the U1 promoter in Fig. 2C). In contrast, when the internal deletions included amino acids 207 to 220 in the Δ6-220, Δ78-220, and Δ157-220 mutants, activation of transcription was lost (Fig. 2B, lanes 6 to 8; Fig. 2C, compare lane 2 with lanes 3 and 4). The Δ6-206 truncation, associated with a deletion between amino acids 224 and 243 (Δ6-206+Δ225-242), as well as the deletion between amino acids 243 and 263 (Δ244-262), led to a protein as active as the parental Staf-Krox-20 (Fig. 2B, compare lane 11 with lanes 12 and 13; Fig. 2C, compare lane 2 with lanes 6 and 7). This truncation analysis demonstrates that the region from 207 to 224 is critical for activation of the tRNA<sup>Sec</sup> and U1 promoters.

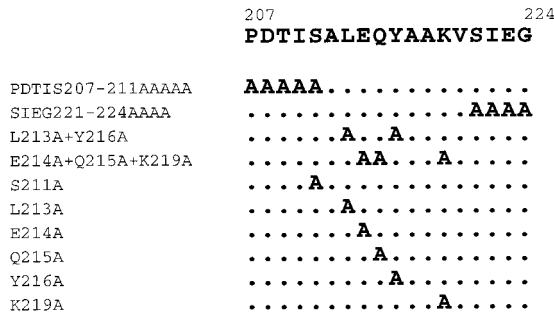
The importance of domain 207 to 224 was further established by examining the effects of deletions within this defined area. Deletions of amino acids 212 to 220 in Staf-Krox-20 (Δ212-220) and Staf-Krox-20 Δ473-600 (Δ212-220+Δ473-600) (Fig. 2A) abolished the transactivation abilities of these effector proteins (Fig. 2B, compare lanes 11 and 15 and lanes 17 and 18; Fig. 2C, compare lane 8 with lanes 9 and 10). Lastly, fusion of the 18-amino-acid domain 207 to 224 to Krox-20 DBD produced protein S207-224K (Fig. 2A) which carried the ability to activate transcription of the tRNA<sup>Sec</sup> (Fig. 2B, com-

pare lanes 19 and 20) and U1 promoters (Fig. 2C, compare lanes 1, 8, and 13). The expression levels and nuclear localization of these chimera were verified by electrophoretic mobility shift assays using nuclear extracts from oocytes injected with mRNAs (data not shown).

These results show clearly that the 18-amino-acid region 207 to 224 of sequence PDTISALEQYAAKVSIEG represents the minimal Staf activation domain for Pol III tRNA<sup>Sec</sup> and Pol II U1 promoters.

**Leu-213 is critical for transactivation activity on snRNA-type promoters.** The region 207 to 224 does not have a preponderance of amino acids, such as glutamines, isoleucines, prolines, or serines-threonines that typify other well-characterized activation domains. To determine residues responsible for activation, mutants were created by alanine substitution mutagenesis in the context of Staf-Krox-20 Δ473-600. Alanine substitutions of amino acids 207 to 211, 221 to 224, and serine 211 (Fig. 3A, PDTIS207-211AAAAA, SIEG221-224AAAA, and S211A, respectively) had a very minor or no effect at all on the ability of the protein to activate the 3E.tRNA<sup>Sec</sup> and 3E.U1 reporters in the *Xenopus* microinjection assay (Fig. 3B). Replacement of the three residues at positions 214, 215, and 219 (Fig. 3A, E214A+Q215A+K219A) produced a twofold drop in the activity of the protein. In stark contrast, the simultaneous replacement of the two residues at positions 213 and 216

**A**



**B**

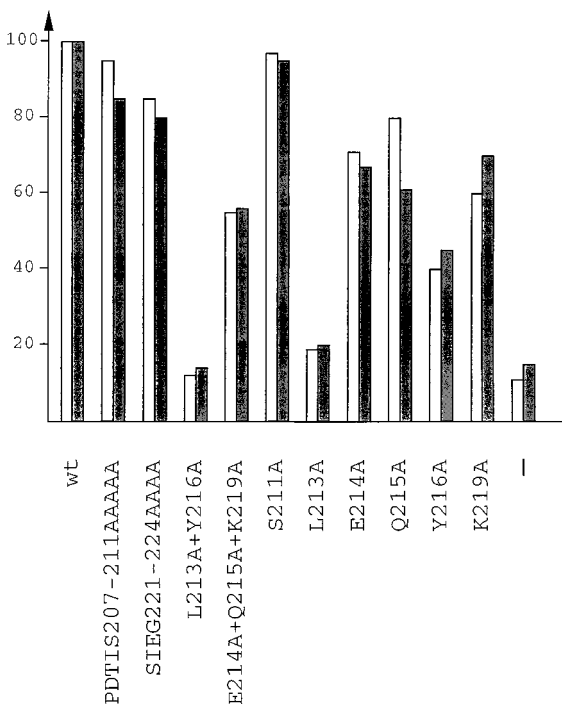


FIG. 3. Identification of amino acids essential for transactivation of snRNA and snRNA-type promoters. (A) The amino acid sequence of the Staf domain 207 to 224 is shown with the changes introduced in the mutants. Dots in the mutant sequences indicate an unchanged residue. (B) Transcriptional activation assays from the Pol II 3E.U1 and Pol III 3E.tRNA<sup>Sec</sup> promoters by the various effectors depicted in panel A. Activity is shown as a percentage of the activity observed with Staf-Krox-20  $\Delta$ 473-600 construct containing the wild-type snRNA activation domain. Activities in microinjections assays with the 3E.U1 and 3E.tRNA<sup>Sec</sup> reporters are shown by solid and shaded bars, respectively. Results are the averages of two independent determinations. Identities of the effector proteins are indicated below each histogram. —, assay without effector.

by an alanine L213A+Y216A (Fig. 3A) completely abolished the transactivating ability of the protein (Fig. 3B). To assess the contributions of Leu-213, Glu-214, Gln-215, Tyr-216, and Lys-219, they were individually mutated to alanines. Whereas changing Glu-214, Gln-215, Tyr-216, and Lys-219 had only a moderate effect, the L213A mutant by itself was severely defective compared to Staf-Krox-20  $\Delta$ 473-600 (Fig. 3B).

These results demonstrate that Leu-213 is essential to the activity of the Staf snRNA transactivation domain.

**Staf contains distinct activation domains for mRNA and snRNA-type promoters.** To determine if the snRNA activation domain of Staf can also activate transcription from an mRNA promoter, we tested the ability of truncated Staf-Krox-20 proteins to enhance the transcription level of the 6E.tkCAT reporter in a microinjection assay. Surprisingly, deletion of residues 212 to 220 in Staf-Krox-20 ( $\Delta$ 212-220) and Staf-Krox-20  $\Delta$ 473-600 ( $\Delta$ 212-220+ $\Delta$ 473-600), corresponding to sequence ALEQYAAKV, had no effect on the transcription level of 6E.tkCAT, while the same mutant effectors failed to activate snRNA-type promoters (Fig. 4, compare lane 2 with lanes 3 and 5). In sharp contrast, deletion of residues 84 to 176 ( $\Delta$ 84-176+ $\Delta$ 473-600; Fig. 2A) in Staf-Krox-20  $\Delta$ 473-600 completely abolished activation from the thymidine kinase (*tk*) promoter of 6E.tkCAT (Fig. 4, compare lanes 2 and 4) but is absolutely innocuous to activation of transcription from the tRNA<sup>Sec</sup> and U1 promoters (Fig. 2B, lanes 11 and 14; Fig. 2C, lanes 8 and 11). This analysis indicated that a region containing residues 84 to 176 is the major contributor to activation of transcription from an mRNA promoter. A fusion of the region containing residues 84 to 176 to the Krox-20 DBD, to yield S84-176K (Fig. 2A), led to efficient activation of transcription from 6E.tkCAT (Fig. 4, compare lanes 2 and 6). However, this same chimeric protein was unable to activate snRNA and snRNA-type promoters (Fig. 2B, lanes 16 and 17; Fig. 2C, lanes 8 and 12). Therefore, this 93-amino-acid region appears to possess an autonomous transactivation capability in the absence of the rest of Staf.

Together, these results demonstrate unambiguously that Staf contains two physically separate and functionally distinct activation domains located in the N terminus of the molecule. The activation domain covering residues 207 to 224 functions solely in the transactivation of Pol II and Pol III snRNA-type promoters. The other activation domain, encompassing amino acids 84 to 176, is restricted to transcriptional activation from mRNA promoters.

**Four repeat units for an mRNA activation domain.** So far, our investigations were carried out with the use of chimeric Stafs bearing the Krox-20 DBD. To obviate the need for this heterologous DBD, which was required to eliminate the endogenous Staf background of the *Xenopus* oocytes, another strategy was taken. *Drosophila* cells, which were shown not to possess a Staf activity, were employed (39). In these cell lines, we could assay only the mRNA activation domain since the literature provides experimental evidence that transcriptional activation of snRNA promoters in *Drosophila* does not proceed similarly to that in vertebrates (8). Full-length Staf or

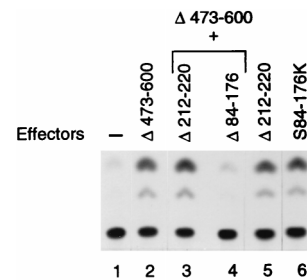


FIG. 4. The mRNA activation domain is distinct from the snRNA counterpart. Representative CAT assays of transcriptional activation from the 6E.tkCAT promoter using extracts from microinjected *Xenopus* oocytes. Identities of the effectors are indicated above the lanes. The effector structures are depicted in Fig. 2A. Expression of the proteins was confirmed by gel mobility shift assays using nuclear extracts from microinjected *Xenopus* oocytes (data not shown).

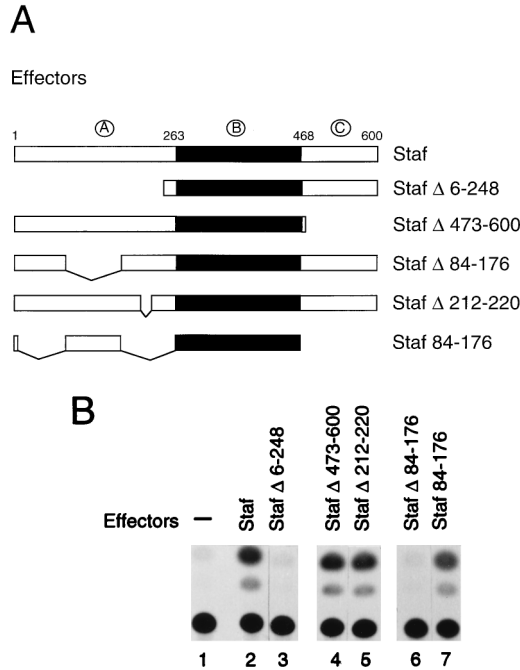


FIG. 5. Transactivation abilities of Staf deletion mutants from an mRNA promoter, assayed in *Drosophila* cells. (A) Schematic structures of the various Staf deletion mutants. The A, B, and C domains of Staf are indicated. The Staf DNA binding domain is represented in black. (B) Representative CAT assays using extracts from *Drosophila* cells transfected with the effectors indicated above each lane.

various deletion mutant versions were expressed under the control of the constitutively active actin 5C promoter (Fig. 5A). The reporter AE.tkCAT contains three Staf binding site AE upstream of the *tk* promoter fused to the CAT reporter gene. Cotransfection of wild-type Staf and reporter plasmid resulted in an efficient transcriptional activation of the CAT gene (Fig. 5B, compare lanes 1 and 2). Deletions of the C-terminal domain C and of amino acids 212 to 220 (Staf Δ473-600 and Staf Δ212-220, respectively) (Fig. 5A) did not reduce the ability of Staf to activate the *tk* promoter (Fig. 5B, compare lane 2 with lanes 4 and 5). In contrast, however, deletion of the N-terminal domain A or an internal deletion between amino acids 83 and 177 (Staf Δ6-248 and Staf Δ84-176, respectively) (Fig. 5A) produced molecules which were unable to stimulate the promoter of the CAT reporter gene (Fig. 5B, compare lane 2 with lanes 3 and 6). The transcriptional activity could be fully recovered with Staf 84-176 (Fig. 5A), a fusion protein consisting of only amino acids 84 to 176 fused to the Staf DBD (Fig. 5B, compare lanes 2 and 7). These results, obtained with *Drosophila* cells, corroborate those already obtained with the *Xenopus* oocyte system with chimeric Staf-Krox-20 proteins.

The 93-amino-acid region (84 to 176) of Staf, which can activate an mRNA promoter as an independent transactivating domain, contains four short repeats of 15 amino acids, with the consensus sequence QAVQLEDG(S/T)TAYI(Q/H)H. The distance between each repeat is 10 to 12 amino acids. The positions of the repeats, previously described (39), are the following: repeat 1 (R1), positions 84 to 98; repeat 2 (R2), positions 109 to 123; repeat 3 (R3), positions 136 to 150; repeat 4 (R4), positions 162 to 176 (Fig. 6A). The presence of four repeat units in the Staf minimal mRNA activation domain represents a novel feature for an activation domain. Therefore, to determine whether they are important for the ability of Staf to

stimulate transcription, internal deletions in full-length Staf were generated and tested again in the *Drosophila* cotransfection assay. Fig. 6B shows the constructs that were made. Deletions from amino acid 77 to amino acids 102 (Staf Δ77-102), 130 (Staf Δ77-130), 156 (Staf Δ77-156), and 220 (Staf Δ77-220), removed the sequences containing R1, R1 and R2, R1 to R3, and R1 to R4, respectively. Truncations between amino acids 102 to 221 (Staf Δ103-220), 130 to 221 (Staf Δ131-220), and 156 to 221 (Staf Δ157-220) eliminated the regions containing R2 to R4, R3 and R4, and R4, respectively. The expression levels and nuclear localization of these mutant proteins were verified by Western blot analysis (data not shown). The normalized values in Fig. 6C show correlation between transcription activity of

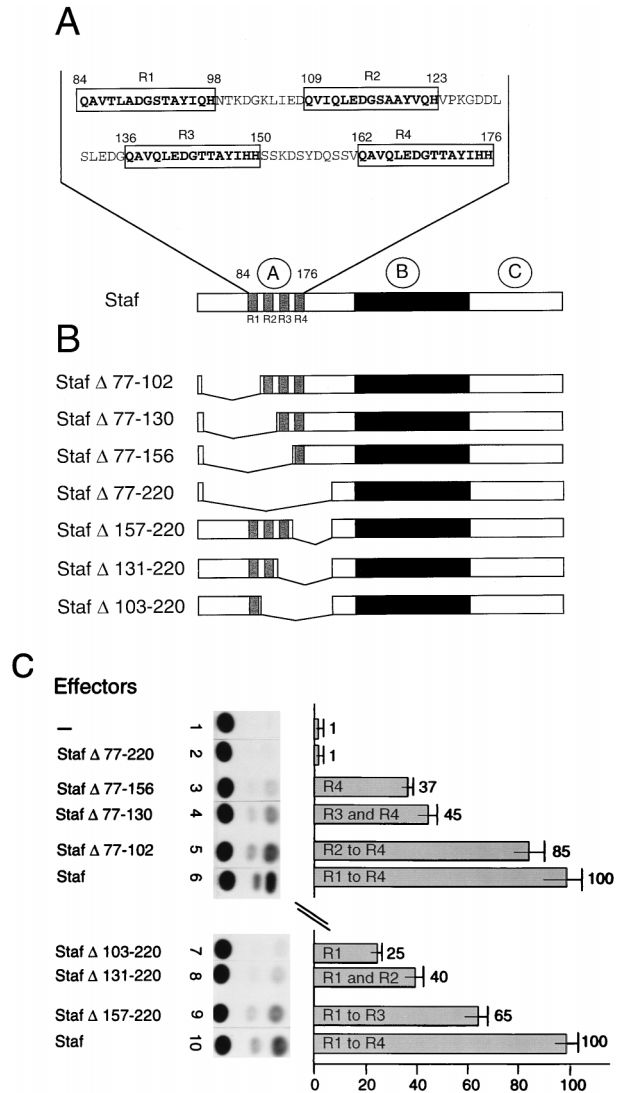


FIG. 6. Maximal transcriptional activation of an mRNA promoter by Staf requires the presence of repeats R1 to R4. (A) Sequence of the Staf mRNA activation domain (amino acids 84 to 176) with the location of repeats R1 to R4. (B) Schematic representation of the seven different effector constructs. The Staf DNA binding domain and the repeats are indicated in the black and gray areas, respectively. (C) Transcriptional stimulation of the AE.tkCAT promoter containing three AE Staf binding sites, assayed in *Drosophila* cells. The autoradiograph from a representative experiment is shown. The names of the effector constructs and the relative transcriptional activities are indicated above the lanes. The values represent the ratio of the CAT activity to that in the absence of effector.

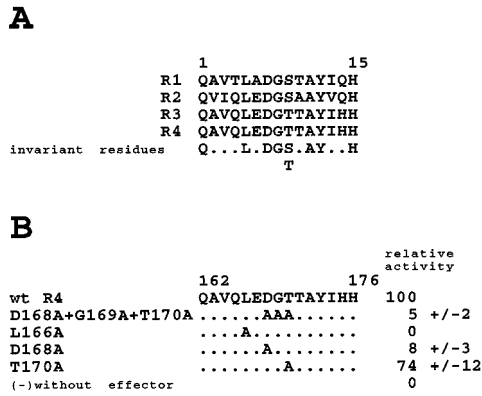


FIG. 7. Alanine substitution mutagenesis of repeat R4 in the Staf mRNA activation domain. (A) Sequence alignments of repeats R1 to R4. (B) Staf Δ77-156 containing repeat R4 with the indicated mutations were tested in *Drosophila* cells for transcriptional activation of a cotransfected AE.tkCAT promoter containing three AE Staf binding sites. The activity of the Staf Δ77-156 construct containing wild-type R4 was defined as 100. Results are the averages (+/- values indicate standard deviations) of three independent determinations. wt, wild type.

the reporter and the number of repeat units. Construct StafΔ77-220, with no repeat, failed to transactivate (Fig. 6C, lane 2). Full activity was progressively restored, however, by the sequential addition of sequences containing the repeat motif (Fig. 6C, lanes 3 to 6 and 7 to 10). The presence of sequences containing repeat R1 (StafΔ103-220) or R4 (StafΔ77-156) resulted in activation levels 25- and 37-fold above the basal level, respectively (Fig. 6C, lanes 7 and 3). When Staf proteins containing the R1 and R2 or R3 and R4 repeat units were tested, transcription levels reached 40- and 45-fold of the basal level, respectively (Fig. 6C, lanes 8 and 4, respectively). More significantly, R1 to R3, but especially R2 to R4, allowed the transcription levels to attain 65- and 85-fold of the basal level (Fig. 6C, lanes 9 and 5, respectively).

Taken together, these results highlight the importance of the four repeat units of Staf to provide maximal transcription activity to mRNA promoters.

Comparison of the four repeats revealed the presence of seven invariant residues located at positions 1, 5, 7, 8, 11, 12, and 15 in each repeat (Fig. 7A). Further, position 9 of each repeat is always occupied by a hydroxylated residue. A number of alanine substitutions were designed in the context of repeat R4 in Staf Δ77-156 to test whether these residues play a role in mediating transactivation. When Asp-168, Gly-169, and Thr-170 were substituted (Fig. 7B, mutant D168A+G169A+T170A), the transactivation properties of the protein on the 3E.tkCAT promoter were dramatically reduced (5% of the R4 wild-type level) (Fig. 7B). Individual alanine replacements of Asp-168 and Thr-170 produced a significant reduction of activity, with the more pronounced effect for Asp-168 (8% of residual level) (Fig. 7B). Strikingly, the alanine substitution of Leu-166 abolished totally the transcriptional activation (Fig. 7B).

From this, we conclude that residues Leu-166, Asp-168, and Thr-170 contribute actively to the activity of the Staf mRNA transactivation domain.

**DISCUSSION**

We have shown previously that Staf plays an important role, not only in the transcriptional activation of snRNA and snRNA-type genes transcribed by RNA polymerases II and III

but also to stimulate expression from an mRNA promoter (32, 38, 39). In this study we have addressed the molecular basis of these pleiotropic effects on transcriptional activity.

Our results summarized in Fig. 8 show that domain A, corresponding to the N-terminal region of Staf, contains two physically and functionally distinct activation domains acting specifically on snRNA-type and mRNA promoters. To our knowledge, this is the first report describing such a feature in a transactivator protein. Based on our work, we propose that the two separate Staf activation domains are involved in two different transactivation mechanisms: one for the specific activation of mRNA promoters and the other for that of snRNA and snRNA-type promoters. Forsberg et al. (11) showed that Sp1 is able to stimulate transcription from mRNA and snRNA promoters. However, it must be pointed out that in this case overlapping domains are involved, while we identified two distinct, specialized domains in Staf.

A number of activators, enhancing transcription from mRNA promoters only, have been shown to contain multipartite activation domains. This is the situation found, for example, in TEF-1, Oct-2, and NGF1-A (19, 36, 46). Interestingly, we have shown in this work that Staf contains one single do-

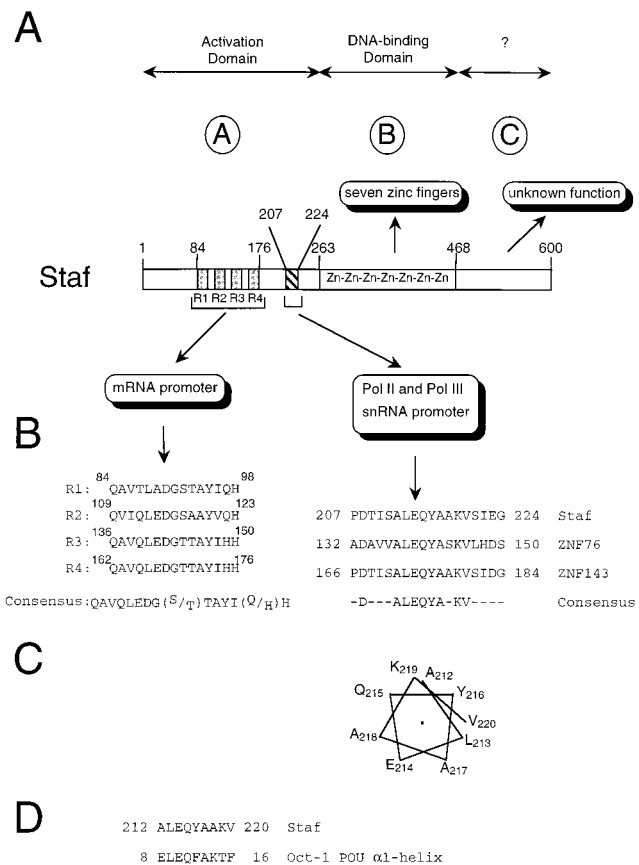


FIG. 8. Summary of Staf subdomains. (A) Schematic representation of Staf shown with delineation of the various subdomains, based on the present and earlier studies. (B) mRNA and snRNA activation domains. Left panel, positions and sequence alignments of the four substructures R1 to R4; a consensus sequence was derived at positions for which at least three of the four repeats contain identical residues. Right panel, sequence comparisons of the snRNA activation domain with regions of ZNF76 and ZNF143 (35, 50). (C) Helical wheel representation of Staf residues 212 to 220. (D) Sequence comparison of Staf residues 212 to 220 with the α-helix-1 from the Oct-1 POU domain. The α1 helix is numbered according to Herr and Cleary (17).

main, necessary and sufficient for optimal transactivation of an mRNA promoter containing a TATA box. This 93-amino-acid activation domain (Staf residues 84 to 176) has a net negative charge and contains 11 and 17% of glutamine and hydroxylated residues, respectively, in a manner analogous to what is found in many other transactivation domains (21, 27, 51). A prominent feature of the Staf mRNA activation domain is its 15-amino-acid motif, repeated four times (Fig. 8B). A related situation was described for the Oct-2 activator, which contains a twofold reiterated 18-amino-acid motif (44). The Staf mRNA activation domain can be divided into subregions containing one, two, three, or four substructures that still have the potential to enhance transcription. This fact suggests that each substructure constitutes a functional unit, even though all four motifs are required to confer optimal transactivation potential. In line with the occurrence of repeated substructures in Staf, previous studies revealed that artificial reiteration of an activation domain can also lead to a significant increase in transcriptional activity (9, 18, 40, 43, 45).

From a mechanistic point of view, involvement of a reiterated motif in the activation process can be apprehended in two ways. In the first one, the affinity of the activator for the basal transcription complex is higher because the number of contacts with the target increases with the number of repeats. Alternatively, redundancy of the interaction surfaces could raise the affinity of the activator by increasing the probability of binding to the target. Structural studies on the VP16 and p53 activation domains suggested that the FXX $\phi$  $\phi$  motif (X stands for any amino acid and  $\phi$  for hydrophobic amino acids) is a general recognition element of the acidic activation domain of TAF<sub>II</sub>31 (22, 52). The Staf mRNA activation domain, though, does not conform to this motif and presents no obvious sequence similarity with the other known activation domains, suggesting that it may constitute the representative of a novel category.

Oct-1 has been shown to activate snRNA promoters with multipartite snRNA activation domains (28, 29, 46). Instead, our study established that Staf performs this function with one single 18-amino-acid region (residues 207 to 224) bearing no sequence similarity to other, previously identified activation domains. The mutational analysis of this domain revealed the prime importance of the 9-amino-acid subdomain of sequence ALEQYAARKV (residues 212 to 220). This subdomain is conserved in the human proteins ZNF76 and ZNF143 (35, 50), which possess significant sequence similarities to Staf (33, 39) (Fig. 8B). This, in addition to the finding that ZNF76 is able to activate transcription of snRNA genes (33), suggests that this new short motif plays a central role in the activation process by constituting an interaction surface within the snRNA transcription complex. Based on protein modeling algorithms, the subdomain composed of residues 212 to 220 is predicted to form an  $\alpha$ -helical region (7, 12). Displaying residues 212 to 220 as a putative  $\alpha$ -helix reveals its amphipathic character, with hydrophilic residues E214, Q215, and K219 distributed on one face (Fig. 8C) and hydrophobic residues L213 and Y216, on the other. From this observation, we propose that hydrophobic protein-protein interactions involving Leu-213 and Tyr-216 are critical in the snRNA gene transcriptional activation by Staf.

A follow-up in the study of domain delineation in a transcriptional activator resides in the identification of the targets that it must contact to activate transcription. Our results show clearly that a short domain of Staf specifically acts as a potent activator of Pol II and Pol III snRNA and snRNA-type gene transcription. The data additionally suggest that in the activation process the molecular target of the snRNA activation domain is a component of the basal Pol II and Pol III snRNA transcription complex. The multisubunit SNAPc complex, also

called the PSE transcription factor PTF, which recognizes specifically the PSE, is the only factor known to be specific and common to Pol II and Pol III snRNA transcription complexes (13, 31, 37, 55). In this respect, it is tempting to speculate that Staf interacts directly or indirectly with SNAPc. The Oct-1 POU protein has been implicated in transcriptional activation of Pol II and Pol III snRNA promoters, as well. In this case, the POU DNA binding domain of Oct-1 not only serves to target the protein to the octamer sequence on the DNA but also is intimately involved in the transcriptional activation process by stabilizing the binding of the basal transcription complex SNAPc (10, 17, 28, 29, 31). It has been proposed that the POU domain does so by recruiting SNAPc to the PSE by direct protein-protein contacts involving the N-terminal part of the POU-specific domain. It is interesting to note that the central part of the Staf snRNA activation domain bears sequence similarity to the  $\alpha$ -helix-1 constituting the N terminus of the POU-specific domain (Fig. 8D). From this, we propose that Staf and Oct-1 recognize the same target in the SNAPc complex.

In conclusion, we have presented experimental evidence for the presence in Staf of two functionally distinct transactivation domains with specific snRNA-type and mRNA gene targets. This represents a novel feature in a transactivator protein and adds an important new dimension to structure-function studies that are being performed with transactivation domains.

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