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Molecular cloning of an essential subunit of RNA polymerase II elongation factor SIII

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ABSTRACT **A transcription factor designated SIII was recently purified from mammalian cells and shown to regulate the activity of the RNA polymerase II elongation complex. SIII is a heterotrimer composed of ≈110-, 18-, and 15-kDa polypeptides and is capable of increasing the overall rate of RNA chain elongation by RNA polymerase II by suppressing transient pausing of polymerase at multiple sites on the DNA template. Here we describe the molecular cloning and characterization of a cDNA encoding the functional 15-kDa subunit (p15) of SIII. The p15 cDNA encodes a 112-amino-acid polypeptide with a calculated molecular mass of 12,473 Da and an electrophoretic mobility indistinguishable from that of the natural p15 subunit. When combined with the 110- and 18-kDa SIII subunits, bacterially expressed p15 efficiently replaces the natural p15 subunit in reconstitution of transcriptionally active SIII. A homology search revealed that the amino-terminal half of the SIII p15 subunit shares significant sequence similarity with a portion of the RNA-binding domain of *Escherichia coli* transcription termination protein ρ and with the *E. coli* NusB protein, suggesting that SIII may be evolutionarily related to proteins involved in the control of transcription elongation in eubacteria.**

The expression of a large fraction of eukaryotic protein-coding genes is controlled by the action of transcription factors that regulate the activity of RNA polymerase II during mRNA synthesis. At least three distinct classes of transcription factors regulate the initiation stage of mRNA synthesis. The general initiation factors TFIIB, TFIID, TFIIE, TFIIF, and TFIIH are sufficient to promote selective binding of RNA polymerase II to the core regions of a large number of promoters and to direct a basal level of transcription (1, 2). DNA-binding transcriptional activators, which include glutamine-rich activators such as Sp1, proline-rich activators such as CTF/NF1, and acidic activators such as GCN4, are not essential for basal transcription but control the rate of initiation by RNA polymerase II from the core promoter (3). Mediators or coactivators are essential for transcriptional activation and appear to mediate the action of DNA-binding transcriptional activators on the basal transcriptional apparatus (4–6).

RNA polymerase II is also a target for the action of transcription factors that regulate the elongation stage of mRNA synthesis (7, 8). These factors fall into at least two functional classes. One class, which includes the type 1 human immunodeficiency virus (HIV-1)-encoded Tat protein and transcription factor SII, has been shown to promote read-through of RNA polymerase II through a variety of transcriptional impediments. Assisted by as yet unidentified cellular proteins, Tat promotes read-through of RNA polymerase II through the HIV-1 attenuation site. The Tat protein functions at least in part through interactions with an RNA hairpin formed by the transactivation response element present in the nascent HIV-1 polyprotein gene transcript. SII is a 38-kDa protein (9) that binds RNA polymerase II and promotes read-through of polymerase through specific attenuation sites in a variety of genes including the histone H3.3, adenovirus 2 major late (AdML), and adenosine deaminase genes. Interaction of SII with the RNA polymerase II elongation complex activates a latent ribonuclease activity that shortens nascent transcripts from their 3′ ends; shortened transcripts remain in the polymerase active site and can be reextended (10). It is believed that this reiterative process of shortening and reextending growing RNA chains is required for efficient read-through of polymerase through blocks to transcription elongation. A number of additional attenuation sites that appear to play a crucial role in controlling gene expression have been identified near the 5′ ends of the *Drosophila hsp70* and human c-*myc* genes. The nature of the transcription factors that enable RNA polymerase II to traverse these sites, however, has not been established.

A second class of transcriptional elongation factors, which includes TFIIF (11, 12) and SIII (13, 14), has been shown to increase the overall rate of RNA chain elongation by RNA polymerase II. In view of evidence indicating that purified RNA polymerase II lacks the capacity to catalyze RNA synthesis *in vitro* at rates greater than 50–100 nucleotides per min (see, for example, ref. 15), whereas mRNA synthesis *in vivo* proceeds at rates of 1200–1500 nucleotides per min (16, 17), transcriptional elongation factors that increase the overall rate of RNA chain elongation may be vital for timely expression of many eukaryotic genes, such as the "mammoth" dystrophin gene, which spans more than 2 megabases (Mb) of chromosomal DNA (18), and the *Drosophila* antennapedia (100 kb), ultrabithorax (70 kb), and ecdysone-induced E74A (60 kb) genes, whose expression is precisely timed during development (19). TFIIF is a multisubunit protein composed of ≈30- and 70-kDa subunits in higher eukaryotes (2) and of 30-, 54-, and 105-kDa subunits in yeast (20). We recently identified and purified mammalian transcriptional elongation factor SIII and demonstrated that it is a heterotrimer composed of ≈110-kDa, 18-kDa, and 15-kDa subunits (13).

In this report, we describe the isolation, structure, and expression of a full-length cDNA encoding the functional 15-kDa subunit (p15) of SIII§. Molecular cloning of the SIII p15 subunit led to the discovery that it shares significant sequence similarity with *Escherichia coli* transcription termination protein ρ and the *E. coli* NusB protein, which both regulate the activity of the *E. coli* RNA polymerase elongation complex (21–23). Here we present these findings, which

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Abbreviation: AdML, adenovirus 2 major late.

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§The sequence reported in this paper has been deposited in the GenBank database (accession no. L29259).

suggest a relationship between transcriptional elongation factors from eubacteria and eukaryotes.

MATERIALS AND METHODS

Isolation of cDNA Encoding the SIII p15 Subunit. SIII was purified to near homogeneity from rat liver nuclear extracts (13). Approximately 300 pmol of the SIII p15 subunit was isolated by reverse-phase HPLC (13). After reduction, S-carboxyamidomethylation, and digestion with trypsin, the resultant mixture was further fractionated by microbore HPLC. Optimal peptides were determined by differential UV absorbance and matrix-assisted laser desorption mass spectrometry (Lasermat; Finnigan-MAT, San Jose, CA) and then submitted to automated Edman microsequencing (see Fig. 1) (24). A partial cDNA encoding residues 51–97 of the SIII p15 polypeptide was isolated by PCR using as primers the sense and antisense degenerate oligonucleotides 5′-CARTTYGC-NGARAAYGARAC-3′ and 5′-GGNGCDATNGGRAAY-TCNGG-3′, which encode residues 8–14 of tryptic peptide II and residues 9–15 of tryptic peptide IV, respectively (R is A or G; M is A or C; Y is C or T; D is A, G, or T; and N is A, C, G, or T). PCR was performed for 30 cycles of 1 min at 94°C, 1 min at 46°C, 2 min at 72°C with 1.5 mM $MgCl_2$, 0.25 mM dNTPs, 2.5 units of *Taq* polymerase, 0.02 A_{260} unit of each primer, and $\approx 6 \times 10^6$ plaque-forming units of a rat liver λgt11 cDNA library (Clontech). PCR products encoding SIII p15 polypeptide sequences were identified by Southern blotting (25), using as probe the 5′ ^{32}P-labeled degenerate oligonucleotide 5′-ACNAAYGARGTNAAYTTYMG-3′, which encodes residues 14–20 of tryptic peptide II, isolated by preparative polyacrylamide gel electrophoresis and subcloned by blunt-end ligation into pBluescript KS(−). Bacteria harboring a recombinant plasmid (pKG1) carrying the partial SIII p15 cDNA were identified by colony hybridization (25) using the same 5′ ^{32}P-labeled degenerate oligonucleotide as probe. cDNAs encoding the complete SIII p15 polypeptide were obtained by screening rat liver and rat brain λZAP II cDNA libraries (Stratagene) with an internally labeled, single-stranded DNA probe synthesized by asymmetric PCR (26) using pKG1 as template. pBluescript SK(−) phagemids containing cDNA inserts were rescued with VCS-M13 interference-resistant helper phage (Stratagene) and sequenced by the dideoxy chain-termination method using a Sequenase kit (United States Biochemical). Two overlapping clones were combined to generate the full-length SIII p15 coding sequence.

Expression of the SIII p15 Protein in *E. coli*. Overexpression of the SIII p15 polypeptide in *E. coli* was accomplished using an M13mpET bacteriophage expression system (27). The entire SIII p15 coding sequence was introduced into M13mpET, which contains the complete pET T7 transcription/expression region (28). A 100-ml culture of *E. coli* strain JM109(DE3) (Promega) was grown to an OD_{600} of 0.6 in SOB medium (25) containing 2.5 mM $MgCl_2$ at 37°C with gentle shaking. Cells were infected with M13mpET carrying the full-length SIII p15 cDNA at a multiplicity of infection of 10–20. After an additional 2 hr at 37°C, cells were induced with 0.4 mM isopropyl β-D-thiogalactoside and the culture was incubated an additional 2.5 hr. Cells were harvested by centrifugation at 2000 × *g* for 10 min at 4°C, and inclusion bodies were prepared as described (29), except that DNase and RNase treatments were omitted. Inclusion bodies were solubilized by resuspension in 2 ml of ice-cold 50 mM Tris·HCl (pH 8.0) containing 6 M guanidine hydrochloride. The resulting suspension was clarified by centrifugation at 50,000 × *g* for 20 min at 4°C.

Reconstitution of Transcriptionally Active SIII Containing the Bacterially Expressed p15 Subunit. One hundred microliters of recombinant SIII p15 protein was diluted with 100 μl of 7.5% acetonitrile in 0.1% trifluoroacetic acid and applied to a Whatman SPE C_8 cartridge preequilibrated with 3.75% (vol/vol) acetonitrile in 0.1% (vol/vol) trifluoroacetic acid. The cartridge was washed with 20% acetonitrile in 0.1% trifluoroacetic acid, and the SIII p15 polypeptide was eluted with 80% acetonitrile in 0.1% trifluoroacetic acid. To reconstitute heterotrimeric SIII, ≈40 pmol of recombinant p15 subunit and ≈5 pmol each of reverse phase-purified p110, p18, and p15 subunits from rat liver (13) were all lyophilized separately until just dry in a Savant Speed-Vac. Dried protein was resuspended in 5 μl of 6.0 M guanidine hydrochloride and left on ice for 30 min. Renaturation mixtures containing 1 μl each of various combinations of SIII subunits were prepared and diluted with 50 μl of buffer containing 40 mM Hepes-NaOH (pH 7.9), 100 mM KCl, 2 mM dithiothreitol, 50 μM $ZnSO_4$, 0.1 mM EDTA, and 10% (vol/vol) glycerol. After a 90-min incubation on ice, renatured proteins were dialyzed for 2 hr against the same buffer lacking EDTA and dithiothreitol.

RESULTS

Isolation of a Full-Length cDNA Encoding the SIII p15 Subunit. Transcription factor SIII was purified to near homogeneity from rat liver nuclear extracts (13). The SIII p15 polypeptide was isolated free of the p110 and p18 polypeptides by reverse-phase HPLC and digested with trypsin. The amino-terminal sequences of four tryptic peptides were determined by sequential Edman degradation (Fig. 1). Using primers selected from regions of low codon degeneracy within peptides II and IV, a partial SIII p15 cDNA was obtained by PCR amplification of a rat liver cDNA library. PCR products encoding p15 sequences were identified by Southern blotting using as probe a degenerate oligonucleotide encoding peptide IV amino acid sequences predicted to be within the PCR product. The appropriate PCR products were subcloned and used as probes to screen rat liver and rat brain cDNA libraries for the full-length SIII p15 cDNA. Several overlapping cDNA clones were isolated, sequenced, and combined to create the full-length SIII p15 cDNA. As shown in Fig. 2, the predicted open reading frame encodes a protein of 112 amino acids with a calculated molecular mass of 12,473 Da and contains sequences encoding all four tryptic peptides. The nucleotide sequences flanking the ATG initiator codon resemble Kozak's consensus sequence for translation initiation sites (30). The TAA translation termination codon is followed immediately by the conventional polyadenylylation signal AATAAA and a poly(A) tail.

Expression of Transcriptionally Active SIII p15 Subunit in *E. coli*. As we demonstrated previously, transcription factor SIII is capable of increasing the overall rate of RNA chain elongation by RNA polymerase II in a reaction strongly dependent on the SIII p15 subunit (13, 14). To confirm that the SIII p15 cDNA encodes the bona fide SIII p15 subunit, the entire open reading frame was subcloned into an M13 expression vector under control of the T7 RNA polymerase promoter, expressed in *E. coli*, and tested for its ability to substitute for the natural SIII p15 subunit in reconstitution of

Peptide	Amino acid sequence
I	LISSDGHEFIVKR
II	AMLSGPGQFAENETNEVNFR
III	VCMYFTYK
IV	YTNSSTEIPEFPIAPEIALELLMAANFLD

FIG. 1. Amino-terminal sequences of p15 tryptic peptides.

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    act agt aac ttc ctc tgg gat caa ata gaa ttt tat aag aac aca

    ATG GAT GGA GAG GAG AAA ACC TAT GGT GGC TGT GAA GGC CCT GAT
  1  M   D   G   E   E   K   T   Y   G   G   C   E   G   P   D

    GCC ATG TAT GTG AAA TTA ATA TCT TCT GAT GGT CAT GAA TTT ATT
 16  A   M   Y   V   K   L   I   S   S   D   G   H   E   F   I

    GTA AAA AGA GAA CAT GCA TTA ACA TCA GGA ACA ATA AAG GCC ATG
 31  V   K   R   E   H   A   L   T   S   G   T   I   K   A   M

    TTG AGT GGT CCA GGT CAG TTT GCG GAG AAT GAA ACC AAT GAG GTC
 46  L   S   G   P   G   Q   F   A   E   N   E   T   N   E   V

    AAC TTT AGA GAG ATC CCT TCA CAT GTG CTA TCG AAA GTG TGC ATG
 61  N   F   R   E   I   P   S   H   V   L   S   K   V   C   M

    TAT TTT ACC TAC AAG GTC CGC TAT ACT AAC AGC TCC ACT GAA ATT
 76  Y   F   T   Y   K   V   R   Y   T   N   S   S   T   E   I

    CCT GAA TTC CCA ATT GCA CCT GAA ATT GCA CTG GAA CTG CTG ATG
 91  P   E   F   P   I   A   P   E   I   A   L   E   L   L   M

    GCC GCG AAC TTC CTA GAT TGT taa ata aaa taa att ata ata aac
106  A   A   N   F   L   D   C

    tgt taa aaa aaa aaa aaa aaa aaa aaa aaa aaa aaa aaa aaa aaa

    aaa aaa aa
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FIG. 2. Sequence of the SIII p15 cDNA and deduced amino acid sequence of p15. Amino acid sequences matching those determined for tryptic peptides are underlined.

transcriptionally active SIII. As shown in Fig. 3, the SIII p15 cDNA directs synthesis in *E. coli* of a protein with an ≈15-kDa apparent molecular mass measured by SDS/PAGE and with an electrophoretic mobility indistinguishable from that of the SIII p15 subunit purified from rat liver (compare lanes 1 and 2). Polyclonal antisera raised against a maltose-binding protein–SIII p15 fusion protein containing amino acids 51–97 of the SIII p15 open reading frame cross-react in Western blots with both the purified rat SIII p15 subunit and recombinant p15 expressed in *E. coli* (Fig. 3, lanes 3 and 4).

As shown in Fig. 4, the bacterially expressed SIII p15 subunit is transcriptionally active. Various combinations of bacterially expressed p15 and the SIII p110 and p18 subunits, purified from rat liver, were renatured and tested for their ability to stimulate the rate of elongation of RNA chains initiated at the AdML promoter. In this experiment, preinitiation complexes were assembled at the AdML promoter, renatured SIII subunits or native rat SIII were added to reaction mixtures, and transcription was initiated by addition of 50 μM ATP, 50 μM GTP, 10 μM CTP, and 1 μM UTP. At these limiting nucleotide concentrations, the rate of RNA chain elongation is very slow; thus, full-length run-off transcripts do not accumulate unless functional SIII is present

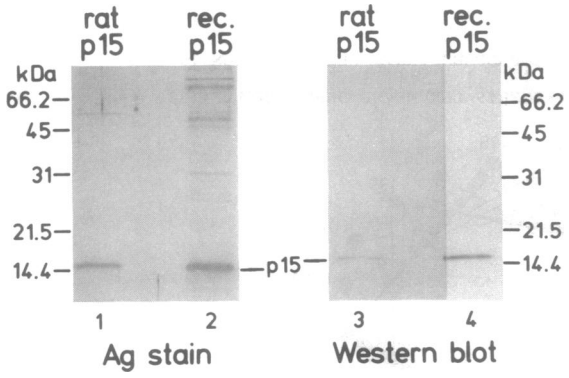

FIG. 3. SDS/polyacrylamide gel and Western blot analysis of bacterially expressed p15. Natural SIII p15 subunit purified from rat liver as described (13) (lanes 1 and 3) or recombinant p15 purified from *E. coli* as described in *Materials and Methods* (lanes 3 and 4) were subjected to SDS/8% PAGE. Lanes 1 and 2, protein was visualized by silver staining. Lanes 3 and 4, protein was transferred to Immobilon-P (Millipore) and analyzed by Western blot as described (31) using rabbit antiserum raised against a maltose-binding protein–p15 fusion protein.

(compare lanes 16 and 18). As shown in lanes 4–13, comparable synthesis of full-length run-off transcripts was observed when transcription was carried out in the presence of native SIII, purified from rat, or of SIII reconstituted with recombinant p15, rat p18, and rat p110. In contrast, almost no stimulation of full-length run-off synthesis was observed when transcription was carried out in the presence of the SIII p18 and p110 subunits, renatured in the absence of recombinant p15 (lanes 1–3). Consistent with our previous results, recombinant p15, in the absence of p110 and p18, failed to stimulate transcription. In addition, all transcription in the presence of either recombinant p15 or purified rat SIII was sensitive to concentrations of α-amanitin that specifically inhibit RNA polymerase II (lanes 15 and 17).

The SIII p15 Subunit Shares Sequence Similarity with a Portion of the RNA-Binding Domain of *E. coli* Transcription Termination Protein ρ. Although results of a TBLASTN search of the GenBank data base indicate that the sequence of the SIII p15 subunit is unique, a FASTA search of the Swiss-Prot data base revealed that an ≈60-amino-acid region of the amino-terminal half of the SIII p15 subunit shares significant sequence similarity with a portion of the RNA binding domain of *E. coli* transcription termination protein ρ (Fig. 5*B*). As determined by the GAP program of the Genetics Computer Group package, the two proteins are 34% identical, 56% similar, and have an alignment score of 6.4 SD within this ≈60 amino acid region of similarity. The ρ factor region similar to p15 includes the carboxyl-terminus of the RNA-binding domain (D. Modrak and J. P. Richardson, personal communication) and sequences believed to be involved in coupling RNA binding and ATPase (22). Interestingly, the SIII p15 region similar to ρ factor shares a short stretch of sequence similarity with *E. coli* transcription elongation factor NusB (Fig. 5*C*). It is noteworthy that both ρ factor and NusB interact with the *E. coli* RNA polymerase elongation complex and are believed to function through interactions with the nascent transcript (21–23).

The SIII p15 polypeptide has several notable structural features besides the region of similarity to ρ factor. Based on its deduced amino acid sequence, p15 has a net negative charge and a predicted isoelectric point of 4.6; it has no noteworthy clustering of acidic or basic residues (Fig. 5*A*). Hydropathy analysis indicates that p15 is largely hydrophilic except for its hydrophobic carboxyl-terminal tail. The Chou–Fasman (34) and Garnier (35) algorithms both predict that the carboxyl-terminal 18 amino acids of p15 have a high probability of forming a short α-helix nucleated by a proline residue at position 94. This region is rich in hydrophobic amino acids that lie on one side of the predicted α-helix (Fig. 5*D*) and therefore has the potential to form a coiled-coil interaction domain analogous to those found in leucine zipper sequences (36, 37). A similar short carboxyl-terminal helix with potential to form a coiled-coil has been shown to play a central role in protein–protein interactions involved in microtubule bundling (38). In addition, the p15 amino terminus has high surface probability and, by the criteria of Leszczynski and Rose (39), may adopt an Ω loop structure. Ω loops, which are found on the surface of a large number of proteins, are proposed in many cases to play important roles in protein function and biological recognition (39). In support of the possibility that the p15 amino terminus is required for its function in transcription, preliminary results suggest that extension of the p15 amino terminus by several amino acids drastically reduces its ability to participate in assembly of functional SIII (data not shown). Based on their predicted structural properties, the p15 amino- and carboxyl-termini are candidates for sites of protein–protein interaction between p15 and other components of the elongation complex.

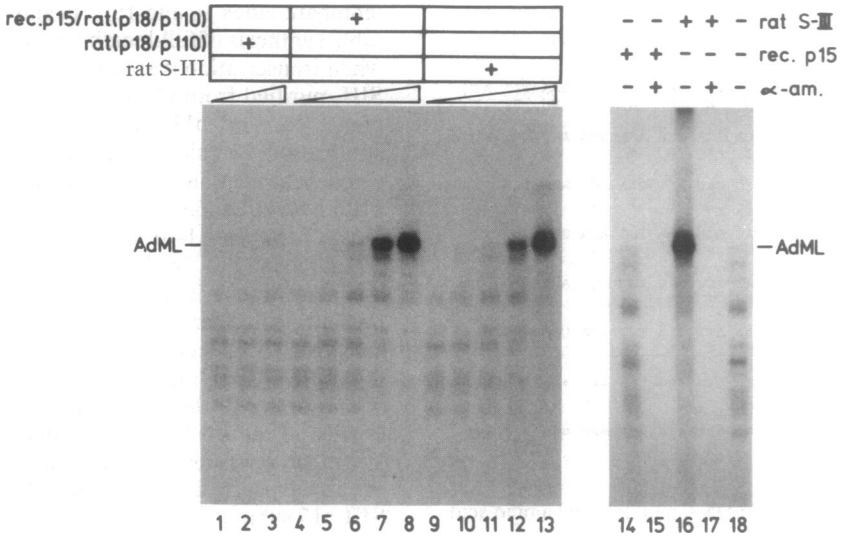

FIG. 4. Transcriptional activity of bacterially expressed, recombinant (rec.) p15. Transcription reactions were carried out as described in the legend of figure 5 in ref. 13. Reaction mixtures contained 1, 3, and 8 μl of a mixture of rat p110 and p18, renatured together as described in *Materials and Methods* (lanes 1–3); 0.03, 0.1, 0.3, 1, and 3 μl of a mixture of recombinant p15 and rat p110 and p18, renatured together as described in *Materials and Methods* (lanes 4–8); ≈30, 10, 3, 1, and 0.3 ng of purified rat SIII (lanes 9–13). In lanes 14–18, reaction mixtures contained α-amanitin (1 μg/ml) and 3 μl of renatured p15 or ≈30 ng of purified SIII, as indicated in the figure.

DISCUSSION

A growing body of evidence suggests that the elongation stage of mRNA synthesis is a major site for the regulation of gene expression in eukaryotes (7, 8). In the process of investigating the mechanism of promoter-specific transcription by mammalian RNA polymerase II, we recently identified and purified a transcriptional elongation factor designated SIII. SIII is a heterotrimer composed of ≈110-, 18-, and 15-kDa polypeptides (13). A variety of evidence argues that SIII functions through a direct interaction with the RNA polymerase II elongation complex to stimulate the overall rate of RNA chain elongation (14). Based on these findings, we speculate that SIII may play a crucial role in controlling the "transit time" of RNA polymerase II through the long stretches of genomic DNA encompassing many eukaryotic genes.

As part of our effort to understand how SIII regulates the activity of the RNA polymerase II elongation complex, we are isolating the genes encoding the SIII subunits. In this

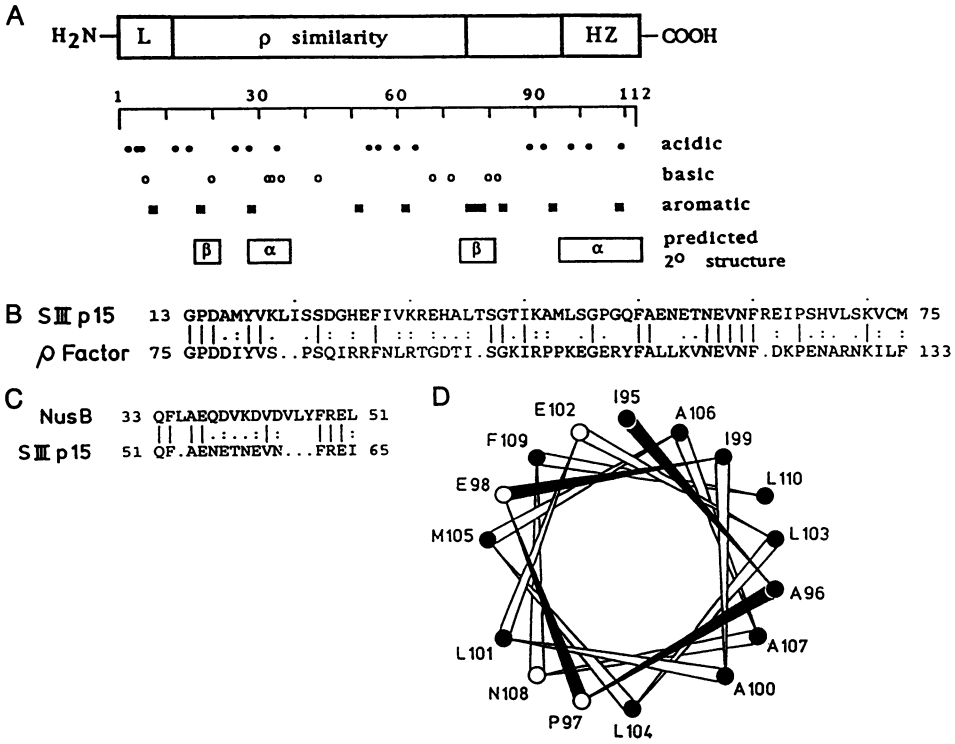

FIG. 5. Structure of the SIII p15 subunit. (*A*) Charge distribution and predicted secondary structure of p15. L, predicted Ω loop; HZ, predicted hydrophobic zipper. (*B* and *C*) Alignments of p15 with *E. coli* ρ factor and NusB. Sequences were aligned using the GAP program of the Genetics Computer Group package (32), using the symbol comparison table of Gribskov and Burgess (33). (*D*) Distribution of hydrophobic amino acids in the predicted carboxyl-terminal α-helix of p15. Filled circles represent hydrophobic amino acids.

report, we describe molecular cloning of a cDNA encoding the functional 15-kDa subunit (p15) of SIII. Analysis of its deduced amino acid sequence reveals that the p15 subunit shares significant sequence similarity with an ≈60-amino-acid region of *E. coli* transcription termination protein ρ as well as with a short stretch of sequence in the *E. coli* transcription elongation factor NusB. ρ factor controls the activity of the *E. coli* RNA polymerase elongation complex by binding to the nascent transcript and causing transcription termination at specific ρ-dependent termination sites (21, 22). ρ factor is an RNA-dependent ATPase and uses the energy of ATP hydrolysis to dissociate RNA polymerase from the DNA template. The ρ-factor region similar to the SIII p15 subunit includes the carboxyl-terminal portion of the ρ factor RNA-binding domain (D. Modrak and J. P. Richardson, personal communication) as well as sequences believed to function in allosteric coupling of RNA binding and ATP hydrolysis (22). NusB, which functions as an antitermination protein, binds to *E. coli* ribosomal protein S10 to form a heterodimeric complex that is capable of binding RNA (23). The NusB–S10 complex interacts with RNA polymerase, nascent RNA, and a set of other elongation factors to promote read-through of polymerase through ρ factor-dependent termination sites. Whether the short NusB sequence similar to p15 participates in RNA binding is presently unknown.

It is not yet clear whether SIII functions through interactions with the nascent transcript. In preliminary experiments, we have not detected a direct, stable interaction of purified SIII with either isolated transcripts or a variety of synthetic RNA homopolymers. Because our evidence suggests that SIII is capable of interacting directly with the RNA polymerase II elongation complex, it is possible that SIII may interact with the nascent transcript in the context of the elongation complex.

In considering the possibility that SIII is an RNA-binding protein, it is important to note that the region of similarity between p15 and ρ factor does not include the entire ρ factor RNA-binding domain; additional ρ factor sequences amino-terminal to the region of similarity with p15 are essential for RNA binding. Moreover, it is clear that NusB binds RNA only when associated with ribosomal protein S10. Based on these observations, it seems unlikely that this region of the SIII p15 subunit is sufficient to constitute an RNA-binding domain. If this region of p15 does participate in RNA binding, it may do so in concert with other regions of p15, with other subunits of SIII, or with RNA polymerase II.

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