A Novel Activity Associated with RNA Polymerase II Elongation Factor SIII

SIII DIRECTS PROMOTER-INDEPENDENT TRANSCRIPTION INITIATION BY RNA POLYMERASE II IN THE ABSENCE OF INITIATION FACTORS*

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General transcription factor SIII, a heterotrimer of 110-, 18-, and 15-kDa subunits, was shown previously to stimulate the overall rate of RNA chain elongation by RNA polymerase II by suppressing transient pausing by polymerase at many sites along DNA templates (Bradsher, J. N., Jackson, K. W., Conaway, R. C., and Conaway, J. W. (1993) J. Biol. Chem. 268, 25587–25593). In this report, SIII is shown to possess the novel ability to direct robust but promiscuous transcription by RNA polymerase II on duplex DNA templates in the absence of initiation factors. Mechanistic studies reveal that SIII promotes RNA synthesis by substantially increasing the efficiency with which RNA polymerase II initiates promoter-independent transcription from the ends of duplex DNA. Remarkably, SIII appears to have a negligible effect on de novo synthesis of end-to-end transcripts. Instead, analysis of reaction products indicates that SIII is capable of promoting a dramatic increase in the ability of RNA polymerase II to extend the 3'-hydroxyl termini of duplex DNA fragments, in a template-directed reaction exhibiting no strong preference for 3'-protruding, 3'-recessed, or blunt DNA ends. Although RNA polymerase II has been shown previously to catalyze primer-dependent transcription, SIII is the first eukaryotic transcription factor found to promote this reaction. Based on these findings, we propose that SIII may suppress transient pausing by RNA polymerase II by helping to maintain the 3'-hydroxyl terminus of the nascent RNA chain in its proper position in the polymerase active site.

A growing body of evidence indicates that the elongation stage of eukaryotic messenger RNA synthesis is a major site for the regulation of gene expression (1, 2). To date, four transcription factors (SII, SIII, TFIIF, and Tat) that regulate the activity of the RNA polymerase II elongation complex have been defined biochemically. On the basis of their mechanisms of action, these four elongation factors have been assigned to two broad functional classes.

One class, which includes SII and Tat, is composed of transcription factors that function as "anti-terminators" to promote readthrough by RNA polymerase II through a variety of transcriptional impediments (3, 4). SII is a 38-kDa protein that binds RNA polymerase II and promotes readthrough by polymerase through specific attenuation sites in such genes as human histone H3.3, adenovirus 2 major late, and adenovirus deaminase, as well as through some DNA-bound proteins and drugs. SII-dependent readthrough by RNA polymerase II through these blocks to elongation is accompanied by a reiterative process of nuclease cleavage and re-extension of portions of the 3'-ends of growing RNA chains. This reiterative process of shortening and re-extending growing RNA chains appears to be an obligatory step in readthrough by RNA polymerase II through SII-sensitive blocks to transcription elongation. Tat is an ~10-kDa protein encoded by the type I human immunodeficiency virus (HIV-1). Assisted by one or more cellular transcription factors, Tat promotes efficient elongation by RNA polymerase II through the HIV long terminal repeat at least in part through interactions with an RNA hairpin present in the transcription response element (TAR) located in the 5'-untranslated region of the HIV-1 polyprotein gene transcript. A number of additional attenuation sites that appear to play roles in regulating gene expression have been identified near the 3'-ends of a variety of genes including the Drosophila hsp70, hsp26, hsp27, α- and β-tubulin, polyubiquitin, glyceraldehyde-3-phosphate dehydrogenase, and human c-myc genes (2, 5). Neither the transcription factors nor mechanisms that regulate passage of RNA polymerase II through these sites have been established.

The second class of transcription factors known to regulate the activity of the RNA polymerase II elongation complex includes TFIIF and SIII, whose primary missions are apparently to boost the overall rate of RNA chain elongation. Because RNA polymerase II purified from many sources is unable to catalyze RNA synthesis on naked DNA templates at rates much greater than 10% of the measured rates of messenger RNA synthesis in vivo (6), elongation factors like TFIIF and SIII are likely to play a vital role in gene expression by reducing the transit time of polymerase over the long stretches of chromosomal DNA encompassing many eukaryotic protein-coding genes. TFIIF is unique among general transcription factors by virtue of its ability to function in both the initiation and elongation stages of transcription (7, 8). In higher eukaryotes, TFIIF is a heterodimer of ~70-kDa (RAP74) and ~30-kDa (RAP30) subunits (8), Saccharomyces cerevisiae TFIIF is a heterotrimer of ~105-kDa, 50-kDa, and 30-kDa subunits; the ~105- and 50-kDa subunits are homologues of RAP74 and RAP30, respectively (9).

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1 The abbreviations used are: HIV, human immunodeficiency virus; AdML, adenovirus 2 major late.
10. We recently purified elongation factor SIII to apparent homogeneity from rat liver nuclear extracts (11). SIII is a heterodimer of ∼110-, 18-, and 15-kDa subunits. Although it is clear that TFIIF and SIII are each capable of interacting directly with the RNA polymerase II elongation complex and strongly stimulating the overall rate of RNA synthesis, their mechanisms of action are poorly understood.

Promoter-specific transcription initiation by RNA polymerase II depends on a set of five general initiation factors referred to as TFIIID (which can be replaced by its TATA box binding subunit TBP), TFIIIB, TFIIIE, TFIIIF, and TFIIH (8). In the absence of these initiation factors, purified RNA polymerase II binds efficiently to single-stranded DNA and initiates transcription, but is not capable of recognizing its promoter or of efficiently initiating transcription on double-stranded DNA templates (12). In the course of studies investigating the mechanism of SIII action, we discovered that it possesses the ability to direct promoter-independent transcription initiation by RNA polymerase II on duplex DNA templates in the absence of initiation factors. Analysis of the products of this reaction revealed that SIII-dependent transcription results from an SIII-induced increase in the ability of RNA polymerase II to extend the 3'-hydroxyl termini of linear duplex DNA templates. Here we describe the properties of this unexpected reaction and its potential significance in the context of current models for the structure and catalytic mechanism of the RNA polymerase II active site.

EXPERIMENTAL PROCEDURES

Materials—Unlabeled ultrapure ribonucleoside 5'-triphosphates and 2',3'-dideoxyribonucleoside 5'-triphosphates were purchased from Pharmacia Biotech Inc. [α-32P]CTP (650 Ci/mmol) was obtained from Amer sham Corp. [γ-32P]ATP (7000 Ci/mmol) was obtained from ICN. α-Amanitin, polyvinyl alcohol (type II), and proteinase K were purchased from Sigma. Bovine serum albumin (Pentex fraction V) was obtained from Nutritional Biochemical Corp., Inc. RNase-free DNase I and recombinant RNasin were obtained from GIBCO BRL. The 28°C reaction mixtures were preincubated at 28°C for 30 min in buffer containing 20 μg Hepes-NaOH, pH 7.9, 20 μg Tris-HCl, pH 7.9, 60 μg KCI, 2 μM dithiothreitol, 0.5 mg/ml bovine serum albumin, 2% (v/v) polyvinyl alcohol, 3% (v/v) glycerol, and 8 units of RNasin. Transcription was initiated by addition of 7 μM MgCl2 and 50 μM ATP, 50 μM GTP, 50 μM UTP, and 10 μM [α-32P]CTP. After incubation at 28°C for an additional 30 min, transcripts were analyzed by electrophoresis through 6% polyacrylamide gels containing 7 M urea.

Nuclease Digestions—The E/H1 and E/H2 SIII-dependent reaction products were synthesized as described above. Radiolabeled products were excised from 6% polyacrylamide gels containing 7 M urea and 0.5 M TBE. Gel slices were crushed and eluted by soaking in 2 M dithiothreitol, 0.14 unit/μl RNasin. The eluted material was divided into 6 tubes. After the addition of 20 μg of yeast-soluble RNA, labeled products were recovered by ethanol precipitation and centrifugation. Individual pellets were resuspended in a final volume of 50 μl of the appropriate nuclease digestion buffer and incubated at 37°C for 10 min with DNase I, RNase H, RNase A, or a mixture of DNase I and RNase A. After proteinase K treatment, digestion products were phenol-chloroform-extracted and analyzed by electrophoresis through 6% polyacrylamide gels containing 7 M urea and 0.5 M TBE. Gel slices were crushed and eluted by soaking in 2 M dithiothreitol.

RESULTS

SIII Promotes Transcription by RNA Polymerase II on Duplex DNA Templates in the Absence of Initiation Factors

In previous studies, we have shown that SIII is an elongation factor that substantially increases the overall rate of RNA chain elongation by RNA polymerase II by decreasing the frequency or duration of transient pausing by polymerase at many sites along the DNA template (11, 24). In the course of investigating the effect of SIII on promoter-specific transcription carried out with RNA polymerase II, TBP, TFIIIB, TFIIIE, and TFIIH, we observed SIII-dependent synthesis of unanticipated RNA products (Fig. 1A). The DNA templates used in these experiments were either the 310-base pair EcoRI to NdeI fragment (E/N) from pDN-AdML, which directs synthesis of a 260-nucleotide runoff transcript from the AdML promoter, or the 197-base pair EcoRI to PvuII fragment (E/P) from pDN-AdML, which directs synthesis of a 130-nt runoff transcript from the AdML promoter. Transcription was carried out for 30 min at 28°C in the presence of 50 μM ATP, 50 μM GTP, 50 μM UTP, and 10 μM [α-32P]CTP. Under these reaction conditions, synthesis of promoter-specific transcripts is independent of SIII, since more than enough time is allowed for completion of full-length runoff products. With both the E/N and E/P templates, the major RNA product synthesized in the absence of SIII was the expected AdML runoff transcript. In the absence of SIII, additional RNA products with electrophoretic mobilities significantly less than those of the AdML runoff transcript and the DNA template were observed following denaturing polyacrylamide gel electrophoresis.
Further investigation revealed that synthesis of SIII-dependent transcripts on a variety of DNA fragments with or without functional class II core promoters neither requires nor is stimulated by the general initiation factors (Fig. 1B, lanes 1–6, Fig. 2, and data not shown). Synthesis of these transcripts depends strongly, however, on both RNA polymerase II and template DNA (lanes 7–14). In addition, synthesis of these transcripts is sensitive to concentrations of α-amanitin that inhibit RNA polymerase II but not mitochondrial RNA polymerase or RNA polymerases I and III, arguing strongly that the RNA polymerase II active site is responsible for SIII-dependent transcription (lanes 11 and 12). Finally, we observe that the pattern of transcripts synthesized in the experiments shown in this figure and in Figs. 3, 4, and 5; EcoRI; H, HindIII; N, NdeI; P, PvuII.

**FIG. 1.** SIII promotes synthesis of unanticipated RNA products. A, runoff transcription assays were performed as described under “Experimental Procedures” in the absence or presence of native rat SIII and the indicated templates. B, SIII-dependent transcription assays were performed as described under “Experimental Procedures” with 50 ng of the indicated template. Where indicated, reactions contained SIII. Template DNA was omitted from the reactions in lanes 9 and 10; RNA polymerase II was omitted from the reactions in lanes 13 and 14; and the reactions in lanes 11 and 12 contained 1 μg/ml α-amanitin. C, SIII-dependent transcription assays were performed as described under “Experimental Procedures” with ~10 ng of the indicated template and ~20 ng (lanes 2 and 7) or 40 ng (lane 3) of native rat SIII or 1 μl (lanes 4 and 8) or 2 μl (lane 5) of recombinant SIII, renatured as described under “Experimental Procedures.” The diagram at the bottom shows the template fragments used in the experiments shown in this figure and in Figs. 3, 4, and 5.

**FIG. 2.** SIII-dependent RNA synthesis requires DNA ends. SIII-dependent transcription assays were carried out as described under “Experimental Procedures” in the presence (lanes 1, 3, 5, and 7) or absence (lanes 2, 4, 6, and 8) of SIII with ~50 ng of uncut pUC18 (supercoiled DNA, lanes 1 and 2), KpnI-digested pUC18 (3′-protruding ends, lanes 3 and 4), SalI-digested pUC18 (5′-protruding ends, lanes 5 and 6), or HindIII-digested pUC18 (blunt ends, lanes 7 and 8) as template.

Further investigation revealed that synthesis of SIII-dependent transcripts on a variety of DNA fragments with or without functional class II core promoters neither requires nor is stimulated by the general initiation factors (Fig. 1B, lanes 1–6, Fig. 2, and data not shown). Synthesis of these transcripts depends strongly, however, on both RNA polymerase II and template DNA (lanes 7–14). In addition, synthesis of these transcripts is sensitive to concentrations of α-amanitin that inhibit RNA polymerase II but not mitochondrial RNA polymerase or RNA polymerases I and III, arguing strongly that the RNA polymerase II active site is responsible for SIII-dependent transcription (lanes 11 and 12). Finally, we observe that the pattern of transcripts synthesized in the presence of recombinant SIII and native rat SIII is similar (Fig. 1C), indicating that the reaction is due to SIII and not to a contaminant in the native SIII preparation.

SIII-dependent RNA Synthesis Requires Free DNA Ends

In an effort to understand how SIII promotes transcription by RNA polymerase II on duplex DNA fragments in the absence of initiation factors, we sought to determine how RNA polymerase II initiates SIII-dependent transcription. To begin with, we considered two possible initiation mechanisms. First, SIII might enable RNA polymerase II to form an open complex and initiate transcription at internal sites within the DNA template in the absence of initiation factors. Alternatively, SIII might promote initiation of transcription at the ends of DNA templates. To distinguish between these possibilities, we compared the efficiency of SIII-dependent transcription on circular and linearized plasmid templates. Circular pUC18 or pUC18 linearized with Sad, KpnI, or HindIII to generate plasmid DNAs with 3′-recessed, 5′-recessed, or blunt ends were used as
templates for transcription reactions carried out in the presence or absence of SIII. As shown in Fig. 2, SIII promotes significant levels of transcription by RNA polymerase II on all three linearized plasmid templates. In contrast, very little SIII-dependent transcription occurred on the uncut plasmid template, indicating that SIII-dependent transcription initiation by RNA polymerase II requires DNA ends. Taken together, these findings suggest that SIII-dependent RNA products are initiated by RNA polymerase II at the ends of DNA templates.

**SIII Promotes Extension by RNA Polymerase II of the 3'-Hydroxyl Termini of DNA Templates**

Previous studies have established that RNA polymerase II is capable of initiating transcription on duplex DNA templates either by initiating RNA chains de novo at the ends of DNA fragments or by extending pre-existing 3’-hydroxyl termini at nicks or gaps in duplex DNA (12). Three lines of evidence indicate that SIII promotes extension by RNA polymerase II of the 3’-hydroxyl termini of DNA templates, leading to the formation of products capable of adopting complex secondary structures.

**Product Analysis—Products of SIII-dependent transcription reactions were analyzed by digestion with DNase I, which degrades both single-stranded and double-stranded DNA, RNase H, which degrades the RNA portion of DNA-RNA hybrids, RNase A, which degrades single-stranded but not double-stranded RNA, or a mixture of DNase I and RNase A. Two predominant products (E/H1 and E/H2) of SIII-dependent transcription on the 100-base pair E/H template (Fig. 3A) were isolated from denaturing polyacrylamide gels and subjected to nuclease digestion. As shown in Fig. 3B, compare lanes 1 and 2 and lanes 7 and 8, both E/H1 and E/H2 were sensitive to digestion with DNase I, arguing that SIII-dependent transcripts are covalently linked to template DNA.

Upon DNase I digestion, the electrophoretic mobility of the E/H1 product in polyacrylamide gels containing 7M urea decreased; the electrophoretic mobility of the DNase I digestion product was slightly greater than that of the denatured template fragment. Because the electrophoretic mobility of double-stranded nucleic acid is greater than that of single-stranded nucleic acid, this result suggests that the E/H1 product has an unusually stable secondary structure that is largely resistant to 7 M urea. Consistent with this possibility, E/H1 was susceptible to digestion by RNase H and by a mixture of DNase I and RNase A, but was largely resistant to digestion with RNase A. Taken together, these results suggest that E/H1 may be a double-stranded, intramolecular “snapback” hybrid of the SIII-dependent transcript and its DNA template; such a product would be synthesized if polymerase initiates its synthesis by extending the 3’-OH of one strand of the DNA molecule and then uses that same DNA strand as template. Regardless of the exact structure of the E/H1 product, our observation that its RNA portion is sensitive to RNase H and, therefore, exists as a DNA-RNA hybrid, argues strongly that synthesis of the E/H1 product is template-directed.

The electrophoretic mobility of the E/H2 product increased upon DNase I digestion. In contrast to the E/H1 product, the E/H2 product was largely resistant to both RNase A and RNase H, suggesting that this SIII-dependent RNA product is capable of forming a double-stranded RNA-RNA hybrid; however, the precise structure of this product is unclear.

As a control for the purity and specificity of the nucleases used in these experiments, the single-stranded RNA products of promoter-specific transcription reactions were digested with the same enzyme preparations used to analyze the E/H1 and E/H2 products. As expected, promoter-specific runoff transcripts synthesized from the AdML promoter were resistant to digestion by RNase I and RNase H, but were sensitive to digestion by RNase A (Fig. 3C).

**Templates Lacking 3’-OH Termini Do Not Support SIII-dependent Transcription—If SIII promotes extension by RNA polymerase II of the 3’-OH termini of template DNA, it should be possible to inhibit SIII-dependent transcription of duplex DNA by addition of deoxynucleotides to the 3’ ends of DNA templates. In the experiment of Fig. 4, the E/N fragment was treated with DNA polymerase I Klenow fragment and a mix-
Mechanism of SIII Action

FIG. 4. SIII-dependent transcription requires a template with a 3'-hydroxyl terminus. A, promoter-specific runoff transcription assays using E/N and ddE/ddN DNA templates were carried out as described under "Experimental Procedures." Reactions in lanes 1, 2, 3, and 4 contained −2, 6, 20, and 60 ng of E/N DNA template. Reactions in lanes 5, 6, 7, and 8 contained −2, 6, 20, and 60 ng of ddE/ddN DNA template. B, SIII-dependent transcription assays were carried out as described under "Experimental Procedures." The reactions in lanes 9 and 10 contained 6 ng and 20 ng of E/N DNA template, respectively. The reactions in lanes 11 and 12 contained 6 ng and 20 ng of ddE/ddN DNA template. To prepare the ddE/ddN template, 10 μg of pDNA-AdML was digested with EcoRI and NdeI. After digestion, 12 units of DNA polymerase I Klenow fragment (Boehringer Mannheim), ddATP (75 μM final concentration), and ddTTP (75 μM final concentration) were added and incubated at 37°C for 60 min. The resulting template fragment was purified from a 15% low melting temperature agarose gel as described under "Experimental Procedures."

The Electrophoretic Mobility of a 5'-32P-Labeled DNA Template Fragment Is Shifted upon SIII-dependent Transcription—If SIII-dependent transcripts are covalently linked to template DNA, the electrophoretic mobility of DNA fragments that serve as templates for SIII-dependent transcription should be altered so that they comigrate with the SIII-dependent transcription products. To test this possibility, the E/H fragment was end-labeled by treatment with T4 polynucleotide kinase and [γ-32P]ATP and used as template in SIII-dependent transcription reactions. When transcription was carried out in the presence of RNA polymerase II, SIII, and unlabeled ribonucleoside triphosphates, a portion of the radiolabeled template co-electrophoresed in denaturing polyacrylamide gels with the E/H2 product (Fig. 5). We could not determine whether a portion of the radiolabeled DNA template also co-electrophoresed with the E/H1 product because the electrophoretic mobilities of the E/H1 product and labeled template fragment were nearly the same; if E/H1 is synthesized at about the same level as E/H2, it would be obscured by the intense band corresponding to the labeled template.

DISCUSSION

It is well established that purified RNA polymerase II is capable of initiating transcription accurately at the promoter-regulatory regions of eukaryotic protein-coding genes in the presence of a set of general initiation factors referred to as TFIIID, TFIIIB, TFIIIE, TFIIF, and TFIIH (8). In the absence of these initiation factors, RNA polymerase II does not initiate at promoters, but is capable of initiating transcription de novo on single-stranded or supercoiled DNA templates and at nicks or ends of duplex DNA (12, 25, 26). In addition, RNA polymerase II has been shown to catalyze template-directed extension of free DNA 3'-hydroxyl termini at sites of single strand nicks in duplex DNA (27–29). Lewis and Burgess (27) have shown that extension of 3'-hydroxyl termini at single-strand breaks in duplex DNA is the predominant reaction when transcription is carried out in the presence of Mg2+, whereas de novo initiations predominate at these sites when transcription is carried out in the presence of Mn2+. In this report, we have shown that general elongation factor SIII possesses the novel ability to promote template-directed extension by RNA polymerase II of 3'-hydroxyl termini of duplex DNA fragments. This reaction depends strongly on the presence of a free 3'-hydroxyl terminus, but does not exhibit a strong preference for 3'-protruding, 3'-recessed, or blunt DNA ends. Interestingly, SIII appears to have a negligible effect on de novo synthesis of end-to-end transcripts initiated by RNA polymerase II on the same DNA fragments (data not shown). Under the conditions used in our assays (7 mM MgCl2, 28°C), de novo initiation at the ends of DNA templates and synthesis of end-to-end transcripts is barely detectable in either the absence or presence of SIII.

What is the relationship between SIII's ability to promote template-directed extension of DNA primers by RNA polymerase II and its ability to suppress pausing by polymerase during DNA chain elongation? It is unlikely that SIII-dependent extension of duplex DNA ends by RNA polymerase II is a physiologically important reaction, because evidence that it occurs in...
vivo is lacking. As suggested by Salzman and co-workers (29), the template-directed addition of ribonucleotides to 3'-hydroxyl termini of DNA by RNA polymerase II may occur in a reaction that mimics formation of the RNA polymerase II ternary elongation complex. In this case, RNA polymerase I would bind the 3'-hydroxyl terminus of DNA in its active site, just as the enzyme binds the 3'-end of an elongating RNA molecule; the polymerase catalytic site for ribonucleotide addition would then use the DNA 3'-hydroxyl terminus as if it were the 3'-hydroxyl terminus of the nascent RNA transcript. In light of this model, our observation that SIII promotes extension by RNA polymerase II of the 3'-hydroxyl termini of DNA primers is consistent with the idea that SIII may facilitate proper positioning of DNA 3'-hydroxyl termini (and, by extension, the 3'-hydroxyl termini of nascent transcripts) with respect to the polymerase catalytic site. If this is correct, the mechanisms by which SIII promotes extension of DNA 3'-ends and suppresses transient pausing by the RNA polymerase II elongation complex could be closely related.

Elongation by RNA polymerase II is an inherently discontinuous process, often punctuated by transient pauses at many sites along DNA templates or by transcriptional arrest at specific DNA sequences referred to as intrinsic arrest sites (1, 30). It has been suggested that transcriptional arrest occurs when the polymerase catalytic site for nucleotide addition slips more than 7 or 8 nucleotides upstream from the 3'-hydroxyl terminus of the nascent transcript (31, 32). The arrested elongation complex is inactive until released from arrest by the action of elongation factor SII, which promotes cleavage of the nascent transcript upstream of its 3'-terminus, thereby creating a new RNA 3'-terminus that is correctly positioned with respect to the catalytic site (3). If transient pausing results when the polymerase catalytic site is only slightly displaced from its position at the 3'-end of growing transcripts, it is possible that SIII promotes efficient RNA chain elongation by ensuring that the catalytic site and 3'-hydroxyl terminus of the nascent transcript are kept in register.

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