The C53/C37 Subcomplex of RNA Polymerase III Lies Near the Active Site and Participates in Promoter Opening*5

George A. Kassavetis1, Prachee Prakash2, and Eunjung Shim
From the Division of Biological Sciences, University of California, San Diego, La Jolla, California 92093-0634

RNA polymerase III (pol III)3 transcribes genes encoding short RNA products that are essential for protein synthesis (e.g. tRNAs, 5 S rRNA), RNA processing (e.g. U6 small nuclear RNA, the RNA subunit of RNase P), protein transport (7SL RNA of the signal recognition particle), and diverse other functions, particularly in higher eukaryotes (1). Pol III is brought to the transcriptional start sites of its genes through interactions with its central 3-subunit initiation factor, TFIIIB (subunits Brf1, TBP, and Bdp1). TFIIIB, in turn, is assembled on DNA upstream of the transcriptional start site in a largely sequence-independent process by its six-subunit assembly factor, TFIIIC. We show that the C53/C37 subcomplex additionally plays a role in formation of the initiation-ready open promoter complex similar to that of the Brf1 N-terminal zinc ribbon domain. In the absence of C53 and C37, the transcription bubble fails to stably propagate to and beyond the transcriptional start site even when the DNA template is supercoiled. The C53/C37 subcomplex also stimulates the formation of an artificially assembled elongation complex from its component DNA and RNA strands. Protein-RNA and protein-DNA photochemical cross-linking analysis places a segment of C53 close to the RNA 3′ end and transcribed DNA strand at the catalytic center of the pol III elongation complex. We discuss the implications of these findings for the mechanism of transcriptional termination by pol III and propose a structural as well as functional correspondence between the C53/C37 subcomplex and the RNA polymerase II initiation factor TFIIF.

1 To whom correspondence should be addressed: Center for Molecular Genetics, University of California, San Diego, 9500 Gilman Dr., La Jolla, CA 92037. Tel: 858-534-2451; Fax: 858-534-7073; E-mail: gak@ucsd.edu.
2 Present address: Synthetic Genomics Inc., 11149 North Torrey Pines Rd., La Jolla, CA 92037.
3 The abbreviations used are: pol, RNA polymerase; TF, transcription factor; ABUTP, 5′-N′)-(p-azidobenzyoyl)-3-aminoallyl]UTP; 3′-O-Me, 3′-O-methyl; HA, hemagglutinin; nt, nucleotide(s); TBP, TATA-binding protein; TEV, tobacco etch virus.

5 This work was supported, in whole or in part, by National Institutes of Health Grant GM18386 (NIGMS).

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–7.

The initial impetus for the analysis that follows was to better understand the function of the 110-residue C11 subunit of pol III. The intrinsic exoribonuclease (proofreading) activity of pol III (9) requires its C11 subunit (7). The amino acid sequences of the C11 N-terminal and C-terminal zinc binding domains are significantly similar to the counterpart segments of the pol II subunit Rpb9, but the C-terminal zinc binding domain of C11 is even more similar to domain III of TFIIS, the extrinsic exoribonuclease-pol II elongation factor. TFIIS domain III contains a zinc binding domain that docks at the entrance to the pol II pore 1 and inserts a β-hairpin into pore 1. Two acidic residues at the tip of this β-hairpin contribute to coordination of a second metal ion for S2-type nucleophilic attack on the RNA phosphodiester bond at the pol II catalytic center (10). The β-hairpin and the two acidic residues at its tip are conserved in C11, and the acidic residues are essential for the intrinsic cleavage activity of pol III (8). Cryoelectron microscopy structural analysis of pol III (11) shows mass density compatible with the N-terminal zinc binding domain of C11, occupying a position on the C128 lobe domain of pol III that resembles the location of the N-terminal zinc binding domain of Rpb9 on Rpb2 in the structure of pol II (12). A mass corresponding to the C-terminal zinc binding domain of C11 was not observed in the pol III electron microscopic structure, but it has been suggested that this domain may bind to the pol III pore 1 (10). However, the pol I counterpart to C11, A12.2, which is also required for exonucleolytic retraction and also contains a TFIIS-like C-terminal zinc binding domain and β-hairpin with the same two acidic
residues at its tip, nevertheless situate both its N- and C-terminal zinc binding domains in Rpb9-manner on the pol I lobe, prompting the suggestion that the pol I A12.2 subunit plays only an indirect role in exonucleolytic retraction (13).

We have approached the question of whether C11 promotes the exoribonucleolytic activity of pol III directly at the catalytic site (as TFIIIS does) or indirectly from its lobe binding site (as does the A12.2 pol I subunit) by probing C11 proximity to the catalytic center of the pol III elongation complex using photochemical RNA-protein and DNA-protein cross-linking methods. The searched-for specific cross-linking to C11 could not be detected; we have instead found that a segment of C53 lies near the RNA 3’ end of the pol III elongation complex. Further analysis indicates that the function of the C53/C37 subcomplex is not confined to transcriptional elongation but that it also facilitates the formation of the open promoter complex, suggesting a possible relationship to TFIIIF (also originally identified as a pol II initiation factor and more recently understood to play an important role in transcript elongation).

**EXPERIMENTAL PROCEDURES**

**Nucleic Acids and Nucleotides—5-[
\(N’\)-(p-Azidobenzoyl)]-3-
aminooallyl]UTP (ABdUTP) was synthesized as described for
ABdUTP (14). The dinucleotide GpA and transcription chain
terminator 3’-methyl-GTP (3’-OMeGTP) were purchased. DNA strands for constructing 3’-overhanging templates and
DNA or RNA primers were purchased and purified by denaturation
PAGE.

ABdUMP-containing DNA for analysis of photochemical cross-linking was synthesized as described (14) with bottom strand DNA primer 3’ ends at register +1 and +9 (supplemental Fig. S5A). 3’-Overhanging DNA templates for transcription were assembled by annealing individual DNA strands and purified by native PAGE, with \(^{32}P\)-labeled markers for the separated strands and duplex DNA. The relatively small quantities of DNA produced in this way were quantified by fluorescence of bound PicoGreen relative to a DNA standard curve. The *SLP4* probe for Km0N0 footprinting was generated by PCR of pTA30 (15) with a 5’-\(^{32}P\)-labeled upstream primer.

**Proteins—**TFIIIC, affinity-purified on oligo (box B +) DNA-Sepharose, and wild type pol III (Mono Q-purified) have been
described (4); quantities are specified as fmol active for binding to
the *SLP4* tRNA gene box B element and for single-round transcription of *SLP4*, respectively. Recombinant wild type Brf1 (N- and C-His\(_6\)-tagged), reference type Brf1Δ366–408 and NΔ68Brf1Δ366–408 (each N-His\(_6\)-tagged), wild type Bdp1 and Bdp1Δ355–372 (both C-His\(_6\)-tagged), and TBP were purified as described or cited (16); quantities are expressed as fmol of protein.

Recombinant C53, C37, and C11 and yeast pol IIIΔ were derived from a library of yeast strains (4) expressing multiply
tagged pol III subunits, with a matching set of pET21-derived vectors for expression of each tagged subunit in *Escherichia coli*. Details of all constructs are available on request. Briefly, two forms of pol IIIΔ were purified from *S. cerevisiae* expressing *Schizosaccharomyces pombe* C11 from a galactose-inducible plasmid promoter in place of the deleted chromosomal copy of *S. cerevisiae* RPC11 (encoding C11). In one strain the chromosomal RET1 gene (encoding C128) additionally encoded an N-His\(_6\)-FLAG\(_3\) tag, and the chromosomal RPO31 gene (encoding C160) was deleted and expressed as an N-HA\(_3\)-tagged protein from a galactose-inducible plasmid promoter (pol IIIΔN); in the other strain the chromosomal RET1 gene additionally contained N-His\(_6\) and C-Myc3 tags with the chromosomal RPO31 gene deleted and expressed from a galactose-inducible plasmid promoter as a C-HA\(_3\)-tagged protein (pol IIIΔC). *S. cerevisiae* strains were derived from the heterozygous diploid yeast knock-out collection (Open Biosystems; background BY4743) with the kanamycin resistance gene replacement cassette (KANMX) replaced by homologous recombination with hygromycin B resistance (HYGMX), nourseothricin resistance (NATMX), or *URA3* (*URA3MX*) cassettes (17, 18) as required; yeast genetic manipulations followed (19). The coding sequences of all of the genes encoding subunits unique to pol III were cloned as SacII-Smal fragments into the pRS313–317 series of yeast centromeric plasmids (20) modified to contain the GAL1 promoter (21) and a Ncol-SacI-Smal-Xhol cassette with epitope tags FLAG\(_3\), HA\(_3\), Myc3, or V5\(_2\) or no tag inserted between Ncol and SacII or between Smal and Xhol sites. Apart from these tags, construction added amino acid residues MPVR to the N termini and PG to the C termini of their respective pol III subunits.

Genes encoding pol III subunits were also cloned into pET21b with a related cassette (Ndel-Ncol-SacII-Smal-Xhol) either with a His\(_6\) tag or a His\(_6\)-TEV protease cleavage tag inserted between Ndel and Ncol; the latter vector was used to generate N-His\(_6\)-TEV-C11 and untagged C11 (upon TEV protease cleavage). C128 cloned into this pET21b-N-His\(_6\) vector with either an N-FLAG\(_3\) or C-Myc3 tag was used as a PCR template for the addition of ~100 bp of N- and C-flanking sequences of *RET1* for transformation and recombination of the PCR product into a yeast strain in which *RET1* was replaced by *URA3MX*, and C128 was expressed as an N-FLAG\(_3\)-tagged protein from the galactose-dependent promoter on pRS313 (HIS3) described above (genotype: *MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 ret1::URA3MX, pRS313:N-FLAG\(_3\)-C128). Recombinants were isolated based on 1) 5-fluoroorotic acid counterselection of the *URA3MX* marker, 2) galactose-independent growth with glucose as carbon source, 3) restoration of the His\(_\text{+}\) phenotype upon subsequent loss of the pRS313 vector, and 4) sequencing of chromosomally integrated FLAG- or Myc-tagged *RET1*. N- or C-HA-tagged RPO31 (encoding C160) was introduced on pRS313 in tandem with the PCR-derived *rpo31::KANMX*, with the flanking sequence from the C160 knock-out strain for recombinational replacement of *RPO31*. *S. pombe* RPC11 was introduced as an untagged (Ncol-Xhol) insert into the pRS316 (*URA3*) galactose-dependent promoter vector along with PCR-derived *rpc11::NATMX* from the C11 knock-out strain.

Pol IIIΔN and pol IIIΔC were purified from 80 g of cells that were lysed with a BeadBeater (Biospec Products) at 33% (w/v)
slurry in 40 mM NaHepes, pH 7.8, 7 mM MgCl\(_2\), 5% (v/v) glycerol, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 0.5 mM NaCl, 1 mM benzamidone, 0.5 mM phenylmethylsulfonfyl fluoride, 1 mM

---

4 G. A. Kassavets, P. Prakash, and E. Shim, unpublished information.
μg/ml each of leupeptin, pepstatin, and aprotinin. Cell debris was removed by centrifugation at 25,000 × g for 30 min, and the supernatant fluid was subjected to ultracentrifugation at 150,000 × g for 90 min. The supernatant was adjusted to contain 20 mM imidazole, pH 7.0, loaded onto a HisTrap HP column (GE Healthcare) and washed successively with 5, 10, and 5 column volumes of the same buffer containing 20, 30, and 40 mM imidazole, respectively. Pol III eluted in the same buffer with 100 mM imidazole. The transcriptional properties of pol IIIΔN and pol IIIΔC were indistinguishable, and they were used interchangeably in this work. Quantities of pol III are approximately specified based on multiple-round transcription of the SUP4 tRNA gene in the presence of excess recombinant C53, C37, and C11 relative to known quantities of purified wild type pol III. Recombinant C53, C37, and C11 were produced in E. coli and purified on Ni-NTA-agarose as described elsewhere for Brf1α-TBPc-Brf1λ (22). Each protein was further purified by size exclusion chromatography on Sephadex G75 in buffer CB+500 (described in the next paragraph). Quantities are specified as nM protein.

Assays—Elongation complexes were formed on 3’-overhang templates for 30 min at 20 °C in 20–40 μl volumes of reaction medium containing 40 mM NaHepes, pH 7.8, 7 mM MgCl2, 3 mM 2-mercaptoethanol, 90 mM NaCl, 0.3–0.5 units/μl placental ribonuclease inhibitor, 100 μM GTP, 100 μM 3’-OMeGTP (unless otherwise specified), 25–50 μM ATP, 1 μM [α-32P]CTP (300–1500 Ci/mmol) or 5 μM CTP, and 100 μM ABUTP or 25 μM UTP. DNA serving as the transcription template was added to 25 nM (Fig. 1C, supplemental Fig. S2), 50 nM (Fig. 1B and supplemental Fig. S1A), 100 nM (Figs. 2, A and B, and 3 and supplemental Fig. S4B), or 170 nM (supplemental Figs. S3 and S4A). Radioactively labeled DNA serving as a probe for footprinting or photochemical cross-linking analyses was added to 0.1 nM (supplemental Fig. S5C), 0.3 nM (supplemental Fig. S5B), or 2.5 nM (Fig. 4 and supplemental Fig. S6). Pol IIIΔA concentrations were 0.1 nM (Fig. 1C and supplemental Fig. S5C), 0.2 nM (Fig. 1B and supplemental Fig. S1A), 0.3 nM (supplemental Fig. S5B), 0.4 nM (Figs. 2, A and B, and 3 and supplemental Figs. S2, S3, and S4B), or 0.5 nM (Fig. 4, and supplemental Figs. S4A and S6). Concentrations of C53, C37, and C11 are specified in the figure legends. When indicated, elongation complexes were separated by size exclusion chromatography on 800-μl Sephacryl S300HR columns (Sephadex G75 superfine for Fig. 1B) equilibrated with buffer CB (20 mM NaHepes, pH 7.8, 3 mM 2-mercaptoethanol, 5% (v/v) glycerol, 0.01% (v/v) Tween 20, 0.1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, 1 μg/ml leupeptin) with 100 mM NaCl (CB+100). Where indicated, 300 μl of buffer CB+500 mM NaCl was loaded onto the column before sample application. Transcription reaction mixtures and column buffers for experiments not involving photoactive RNA or DNA contained Tris chloride, pH 8.0, in place of NaHepes and dithiothreitol in place of 2-mercaptoethanol. UV irradiation and nuclease treatment followed (14), except that 0.5 units/μl micrococcal nuclease replaced S1 nuclease.

Exonuclease III footprinting was performed by adding an equal volume of buffer CB containing 2 units/μl exonuclease III to reaction samples in buffer CB+100 containing 1 mM MgCl2. Digestion was stopped after 10 min at 25 °C, and samples were processed for gel electrophoresis as described (23). Factor-dependent preinitiation complex formation and transcription was performed in the above reaction buffer containing 100 μg/ml bovine serum albumin (with 5 μg/ml poly(dG-dC):poly(dG-dC) added for the experiments shown in Fig. 6 and supplemental Fig. S1B). Preinitiation complexes were formed for 60 min at 20 °C with 2.5 nM each of the 3 TFIIIB subunits, 1.4 nM TFIIIC (for SUP4 transcription in Figs. 6 and 7 and supplemental Fig. S1B) and 0.2 nM wild type pol III, 0.5 nM (Fig. 6) or 0.15 nM (Fig. 7 and supplemental Figs. S1B and S7) pol IIIΔ on the following DNA templates: 2.5 nM SNR6 gene plasmid pLY1855 (24) (for the experiments shown in Fig. 7 and supplemental Fig. S7); 2.5 nM concentration of a 380 bp PCR SUP4 gene fragment from plasmid pTA30 (15) (for supplemental Fig. S1B); 0.5 nM concentration of a 239-bp pTA30-derived fragment, 5’-32P-labeled at bp −78 (relative to the start site of transcription as +1; Fig. 6). Concentrations of C53, C37, and C11 are specified in the figure legends. For KMnO4 footprinting of elongation complexes, ATP, CTP, UTP, and 3’-OMeGTP (to 100 μM each) was added for 4 min to preformed initiation complexes. KMnO4 was added to 20 mM for 30s, the reaction was stopped, and samples were processed as described (25). Multiple-round transcription was allowed to proceed for 30 min by the addition of ATP, CTP, and GTP to 200 μM and [α-32P]UTP (15 cpm/fmol) to 25 μM. Reactions were stopped, and samples were processed as described (23).

Artificially assembled elongation complexes (Fig. 5) were formed in 20 μl of reaction buffer containing 40 mM NaHepes, pH 7.8, 3 mM 2-mercaptoethanol, 1 mM MgCl2, 0.5 units/μl ribonuclease inhibitor, with 50 mM (panel B) or 70 mM (panel C) NaCl. For the RNA-protein cross-linking analysis of elongation complexes in panel C, 6 pmol each of the 10-mer RNA and transcribed DNA strand were annealed, and 5 fmol of pol IIIΔ was added for 10 min at 25 °C followed by 12 pmol of non-transcribed DNA strand and incubation was continued for 10 min at 30 °C. The same procedure was followed for analysis of transcript elongation in panel B with 1.2 fmol of pol IIIΔ and 0.9 pmol of N-Hisα-tagged C53 and C37 and with quantities of RNA and DNA strands specified (in pmol) above each lane. A nucleotide mixture providing 0.5 μM [α-32P]CTP (500 Ci/mmol for panel B or 2500 Ci/mmol for panel C), 25 μM 3’-OMeGTP, and 2.5 μM UTP (panel B) or 40 μM ABUTP (panel C) was added for 10 min at 30 °C. For the experiment shown in panel B, RNA was processed for electrophoresis as cited above. For the cross-linking analysis shown in panel C, 1 pmol each of N-Hisα-tagged C53 and C37 was added for an additional 5 min at 30 °C before UV irradiation and chromatography on Sephacryl S300 as described for Fig. 2B; successively eluting 60-μl fractions from the 800-μl volume column were analyzed (as indicated at the top of Fig. 5C).

RESULTS

We set out to determine directly whether the C11 pol III subunit, like TFIIIS, inserts into the pol III pore 1 (10) and into the catalytic center by seeing whether it was able to form a cross-link to a photoactive nucleotide (4-S-UMP) inserted at or near the 3’ end of the nascent transcript. This approach has
Dual Roles of the Pol III C53/C37 Subcomplex

been successful for detecting TFIIS at the catalytic center of pol II, albeit at low cross-linking efficiency (1–2% relative to the Rpb1 and Rpb2 pol II subunits) (26). We found that 4-S-UTP was inefficiently incorporated by wild type pol III; weak 4-S-UMP-dependent cross-linking to the C160 and C128 subunits of pol III and to a protein approximately the size of the C53 subunit (~50 kDa) was detected (data not shown). We reasoned that poor incorporation of 4-S-UMP was due to C11-mediated proofreading (27) responding to the anomalous base pairing of 4-S-U. Loss of cross-linking had also been observed when both TFIIS and Mg^{2+} were added to pol II elongation complexes containing 4-S-U near the nascent RNA 3′ end (26).

In response to these findings, Pol III was purified from S. cerevisiae expressing S. pombe C11 in place of S. cerevisiae C11. This substitution leads to the loss of the C11, C53, and C37 pol III subunits during purification. The enzyme lacking these three subunits, pol IIIΔ, is defective in transcriptional termination and exoribonucleolytic proofreading. E. coli-expressed C11 restores the intrinsic RNA cleavage activity to pol IIIΔ, and the recombinant C53/C37 subcomplex restores efficient termination (8). The exonucleolytic retraction and termination deficits of a pol IIIΔ preparation and the ability of our recombinant C11, C53, and C37 to restore these functions are documented in supplemental Fig. S1.

The DNA initially chosen for incorporation of a photoactive UMP analogue into the nascent transcript (Fig. 1A) contains a 4-nt 3′-overhang at one end for factor-independent transcription (28) initiating with the dinucleotide primer GpA followed in turn by a run of 15 AMP residues, CMP (32P-labeled), and the photoactive UMP analogue. We expected the initial run of weakly base-pairing A residues to result in reiterative slippage during repetitive addition of AMP to the initially synthesized RNA, generating a cluster of transcripts differing in length but with identical 3′ ends (as previously encountered with E. coli RNA polymerase and also seen to occur with pol III (29, 30)). The array of nascent transcripts contained within elongation complexes formed in the absence of UTP is shown in Fig. 1B, lane 1. That their size heterogeneity resulted from slippage was confirmed by walking the entire cluster of bands upon the addition of UTP (lane 2) or UTP + ATP (lane 3; note that most of the complexes in lane 3 extended to C27 instead of A26 due to misincorporation of UMP and the lack of proofreading by pol IIIΔ (27) and that ~20% of complexes did not chase).

Transcription initiating at 3′-overhanging DNA ends can generate RNA:DNA hybrid products (31). This was not observed with the template shown in Fig. 1A; the 40-nt RNA of a 3′-OMeGMP-arrested pol III elongation complex (Fig. 1C) was resistant to digestion by RNAse H and sensitive to degradation by RNase A, whereas the same elongation complex formed with only the template DNA strand (generating a DNA:RNA hybrid duplex product) was RNase H-sensitive and RNase A-resistant (supplemental Fig. S2).

Unlike 4-S-UMP (or 5-I-UMP), the photoactive analogue ABUMP generated efficient protein cross-linking to C160 and C128 when incorporated near (4 nt upstream of) the nascent RNA 3′ end (data not shown). However, incorporation of tandem ABUMP residues was inefficient (data not shown) and prevented further incorporation (Fig. 1C, compare lanes 2 and 3). A trace level of the 3′-OMeG40-arrested transcript was also observed in the absence of UTP (lane 1) and is interpreted as due either to misincorporation of CMP in place of UMP by error-prone pol IIIΔ or to the presence of trace UTP (the natural deamination product of CTP) in the [α-32P]CTP. Incorporation of tandem 5-p-azidophenacylthio-CMP or -UMP residues has likewise been shown to be inefficient and to prevent further transcript elongation by E. coli RNA polymerase (32).

We initially examined whether one could detect cross-linking of C53, C37, or C11 to ABUMP incorporated at the RNA 3′ end of the pol IIIΔ elongation complex. Pol IIIΔ transcription complexes were chromatographed on Sephacryl S300 to remove unincorporated labeled NTPs and released RNA products (schematically diagrammed in supplemental Fig. S3); when C53 and C37 were added before UV irradiation, a cross-linked product (schematically diagrammed in supplemental Fig. S3) was detected (data not shown). We reasoned that poor incorporation of 4-S-UMP was due to C11-mediated proofreading (27) responding to the anomalous base pairing of 4-S-U. Loss of cross-linking had also been observed when both TFIIS and Mg^{2+} were added to pol II elongation complexes containing 4-S-U near the nascent RNA 3′ end (26).

In response to these findings, Pol III was purified from S. cerevisiae expressing S. pombe C11 in place of S. cerevisiae C11. This substitution leads to the loss of the C11, C53, and C37 pol III subunits during purification. The enzyme lacking these three subunits, pol IIIΔ, is defective in transcriptional termination and exoribonucleolytic proofreading. E. coli-expressed C11 restores the intrinsic RNA cleavage activity to pol IIIΔ, and the recombinant C53/C37 subcomplex restores efficient termination (8). The exonucleolytic retraction and termination deficits of a pol IIIΔ preparation and the ability of our recombinant C11, C53, and C37 to restore these functions are documented in supplemental Fig. S1.

The DNA initially chosen for incorporation of a photoactive UMP analogue into the nascent transcript (Fig. 1A) contains a 4-nt 3′-overhang at one end for factor-independent transcription (28) initiating with the dinucleotide primer GpA followed in turn by a run of 15 AMP residues, CMP (32P-labeled), and the photoactive UMP analogue. We expected the initial run of weakly base-pairing A residues to result in reiterative slippage during repetitive addition of AMP to the initially synthesized RNA, generating a cluster of transcripts differing in length but with identical 3′ ends (as previously encountered with E. coli RNA polymerase and also seen to occur with pol III (29, 30)). The array of nascent transcripts contained within elongation complexes formed in the absence of UTP is shown in Fig. 1B, lane 1. That their size heterogeneity resulted from slippage was confirmed by walking the entire cluster of bands upon the addition of UTP (lane 2) or UTP + ATP (lane 3; note that most of the complexes in lane 3 extended to C27 instead of A26 due to misincorporation of UMP and the lack of proofreading by pol IIIΔ (27) and that ~20% of complexes did not chase).

Transcription initiating at 3′-overhanging DNA ends can generate RNA:DNA hybrid products (31). This was not observed with the template shown in Fig. 1A; the 40-nt RNA of a 3′-OMeGMP-arrested pol III elongation complex (Fig. 1C) was resistant to digestion by RNAse H and sensitive to degradation by RNase A, whereas the same elongation complex formed with only the template DNA strand (generating a DNA:RNA hybrid duplex product) was RNase H-sensitive and RNase A-resistant (supplemental Fig. S2).

Unlike 4-S-UMP (or 5-I-UMP), the photoactive analogue ABUMP generated efficient protein cross-linking to C160 and C128 when incorporated near (4 nt upstream of) the nascent RNA 3′ end (data not shown). However, incorporation of tandem ABUMP residues was inefficient (data not shown) and prevented further incorporation (Fig. 1C, compare lanes 2 and 3). A trace level of the 3′-OMeG40-arrested transcript was also observed in the absence of UTP (lane 1) and is interpreted as due either to misincorporation of CMP in place of UMP by error-prone pol IIIΔ or to the presence of trace UTP (the natural deamination product of CTP) in the [α-32P]CTP. Incorporation of tandem 5-p-azidophenacylthio-CMP or -UMP residues has likewise been shown to be inefficient and to prevent further transcript elongation by E. coli RNA polymerase (32).

We initially examined whether one could detect cross-linking of C53, C37, or C11 to ABUMP incorporated at the RNA 3′ end of the pol IIIΔ elongation complex. Pol IIIΔ transcription complexes were chromatographed on Sephacryl S300 to remove unincorporated labeled NTPs and released RNA products (schematically diagrammed in supplemental Fig. S3); when C53 and C37 were added before UV irradiation, a cross-linked (i.e. radioactively tagged) ~60-kDa protein appeared in addition to the C160 and C128 subunits of pol III (Fig. 2A, lane 2). This ~60-kDa protein was positively identified as C53 by substituting a larger FLAG epitope-tagged version (lane 3). Little or no effect on C53 cross-linking was observed when pol IIIΔ elongation complexes were chromatographed on Sephacryl S300.
through a zone of 0.5 M NaCl to reduce the potential for non-specific re-binding of released RNA to pol III during chromatography (supplemental Fig. S3). The addition of C53 and C37 to the pol III/H9004 elongation complex and UV irradiation before Sephacryl S300 chromatography demonstrated that the cross-linked C53 is associated with the elongation complex (Fig. 2, panels B and C) as evidenced by the nearly perfect co-elution of cross-linked C160, C128, and C53 (panel B), well removed from excess unbound C53/C37 subcomplex and the DNA template (the profile for C37 is omitted for clarity in panel C). The C11 subunit was also cross-linked to RNA in the Sephacryl S300-purified elongation complex (Fig. 2A, lane 5). However, whereas C53 cross-linking to more S300-included fractions progressively diminished, C11 cross-linking increased (data not shown). Moreover, an experiment (performed in parallel with the analysis in panel B) in which C11, C53, and C37 were added to the elongation complex before UV irradiation and Sephacryl S300 chromatography showed no evidence of cross-linked C11 co-eluting with pol III/H9004 (data not shown).

These observations suggested the possibility that the observed C11 cross-linking might merely reflect its direct binding to low levels of released RNA and be unrelated to the structure of the pol III elongation complex. That C53 and C37 bind and cross-link directly to purified ABU-containing RNA is shown in Fig. 3. Both C53 and C11 cross-linked to free RNA (first lane) along with a protein corresponding in size with C37. Single-
stranded DNA with the same sequence was ineffectual as a competitor for binding C53 (at 100-fold molar excess over C53) when added with the labeled RNA (second lane), whereas tRNA competed effectively in both simultaneous (third lane) and sequential (fifth lane) addition formats. Poly(A) RNA was an effective competitor also (data not shown), indicating that C53 preferentially binds to RNA. Unlabeled tRNA (third and fifth lanes) and single-stranded DNA (second lane) were effective competitors for UV-mediated cross-linking of labeled RNA to C11. TFIIIS likewise contains a DNA and RNA binding domain, but it is masked in the full-length protein (33).

UV-cross-linking of C53 and C11 to free RNA warranted further examination of the specificity of RNA cross-linking to C53 in the transcription complex. We considered two ways in which nonspecific binding of C53 to RNA might generate the appearance of cross-linking to the RNA 3′ end within the elongation complex (Fig. 2 and supplemental Fig. S3). The first alternative is that co-chromatography of cross-linked C53 with pol IIIΔ in Fig. 2B could result from stable binding of the UV-cross-linked, nonspecific RNA-C53 complex to pol III. This was assessed directly by adding RNA-cross-link C53 to pol III before Sephacryl S300 chromatography (supplemental Fig. S4A). Although some cross-linked C53 did co-elute with pol III, most of it emerged from the column in later (included) fractions. These findings strongly argued against cross-linked C53 in the Fig. 2 experiment representing adventitiously bound RNA adduct, as the latter would have had to vastly out-compete RNA-free C53/C37 subcomplex for binding to limiting pol IIIΔ.

We also considered the possibility that the photoactive nascent RNA residue responsible for C53 cross-linking in the more stringently purified elongation complex (supplemental Fig. S3) is not located at the catalytic center but in the RNA exit channel of a minor fraction of elongation complexes reading through and extending to G40 (Fig. 1C, lanes 1 and 2). We addressed this possibility by modifying the transcription unit shown in Fig. 1A, replacing AAA at bp 24–26 with TGG (supplemental Fig. S4A). Transcription analysis indicated that in the presence of ABUTP few if any complexes extended an RNA chain to bp 24 and that most of it emerged from the column in later (included) fractions. Because C53 appeared to cross-link to RNA near the catalytic site and because cross-linking to the transcribed DNA strand had not been examined previously, we probed C53 cross-linking to ABdU in the transcribed DNA strand near the catalytic center of our elongation complex. The 3′-overhang DNA template shown in supplemental Fig. S3 was modified to allow incorporation of ABdU at register 1−/−2 or +8/+9 (supplemental Fig. S5A). C53/C37-supplemented Sephacryl S300-purified elongation complexes showed C53 cross-linking at register +8/+9 and −1/−2 and C37 cross-linking at register +8/+9 (supplemental Fig. S5B). Analysis of protein-DNA cross-linking of control samples in which ATP was omitted from the NTP mixture used to form elongation complexes (data not shown) indicated that the small fraction of transcription complexes that failed to initiate (as evidenced by electrophoretic mobility shift analysis) might contribute to C53 cross-linking to the −1/−2 DNA probe. Modest levels of poly(dl-dC):poly(dl-dC) were found to prevent nonspecific binding and cross-linking of pol III and C53 to the −1/−2 probe (supplemental Fig. S5C).

Accordingly, for the experiment shown in Fig. 4, elongation (+ATP) and control nonspecific (no ATP) complexes were formed with pol IIIΔ and DNA probe −1/−2 followed by treatment with 0.5 M NaCl and poly(dl-dC) before chromatography on Sephacryl S300. Individual fractions were supplemented with additional poly(dl-dC) before the addition of C53/C37 and UV irradiation. C53 was found to cross-link to DNA in fractions containing the elongation complex and in constant proportion to C160 as well as C128 in the trailing excluded peak fractions (fractions 3−5, left panel) but not to the bulk of the DNA probe, which eluted with the more included fractions 7 and 8. This cross-linking required the formation of the stable elongation complex as only trace levels of cross-linking to C160, C128, and C53 were discernible in the corresponding excluded fractions.

FIGURE 4. C53 cross-links to the transcribed DNA strand of the pol IIIΔ elongation complex at register 1−/−2. Elongation complexes (+ATP) or mock complexes (no ATP) were formed with the −1/−2 photoactive (ABdUMP) and radioactive DNA probe as described under “Experimental Procedures.” Poly(dl-dC):poly(dl-dC) (to 40 ng/μl) and NaCl to 0.5 M were added before chromatography on Sephacryl S300 equilibrated with buffer CB supplemented with 7 mM MgCl2, with an upper zone of the same buffer containing 0.5 M NaCl. 30-μl volume fractions were taken; to each, poly(dl-dC):poly(dl-dC) was added to 20 ng/μl followed by 30 nM N-His6-C53, 30 nM N-His6-C37, and 30 nM N-His6-TEV-C11. After incubation for 10 min, samples were UV-irradiated and prepared for analysis, as described under “Experimental Procedures.” DNA cross-linked C160, C128, and C53 are identified at the left side. The asterisks at the right identify cross-linked proteins that are not pol III subunits.
in the absence of productive initiation (the no-ATP control; right panel).

We noted that, whereas cross-linking of C160 and C128 to the $-1/-2$ probe was maintained when the polyanion heparin was added to the elongation complex (cf. Ref. 34), C53 cross-linking was heparin-sensitive. DNA-cross-linking of the C53 subunit of elongation complexes formed with highly purified wild type pol III was also found to be heparin-sensitive (data not shown). The implications of this heparin sensitivity are discussed below.

We next examined the possibility that cross-linking of C53 to DNA and RNA near the catalytic center of the elongation complex might be due to backward translocation of the transcription bubble (backtracking (38)), which shifts the DNA:RNA duplex upstream and extrudes the melted RNA 3' end toward or into pore 1, with register +1 of the transcribed strand concomitantly reassociating with the non-transcribed strand. We used exonuclease III ($3' \rightarrow 5'$) digestion from the downstream DNA end of the elongation complex to locate the downstream-facing edge of pol III (F) relative to the RNA 3' end (C, the catalytic center). For *E. coli* RNA polymerase, the F–C separation for non-backtracked elongation complexes is 18 bp, and F–C ≤ 16 bp indicates backtracking (38). We found that the pol III elongation complex formed on a template nearly identical to that used for RNA cross-linking (supplemental Fig. S4A) primarily generated F–C distances of 18 and 19 bp with weaker exo III arrest sites corresponding to F–C distances of 20 and 17 bp (supplemental Fig. S6). A background of incomplete exo III digestion products in the absence of pol III limited the ability to exclude the existence of a small fraction of extensively backtracked pol III complexes. We judge that the 18–19-bp F–C separation reflects the non-backtracked state of the pol III elongation complex. This is consistent with preferential cross-linking of C128 relative to C160 to ABdUMP incorporated at register $-1/-2$ of the transcribed strand (Fig. 4) as well as the relative C128-C160 cross-linking efficiency for ABUMP incorporated at the RNA 3' end (supplemental Fig. S3), which matches the Rpnb2-Rpb1 cross-linking efficiency ratio of the pol II elongation complex with ABdUMP incorporated in the transcribed strand at register −2 and is readily rationalized on the basis of the structure of the pol II elongation complex (39). Extensive (i.e. 6–10 bp) backtracking should generate preferential cross-linking to C160 (Refs. 34 and 39; see also probe +8/+9 in supplemental Fig. S5). Thus, in sum, the preceding experiments strongly support the conclusion that C53 is located within cross-linking distance of the 3' end of the nascent transcript and the transcribed DNA strand at and near the catalytic center.

The other path taken was to limit the extent of, and potential for, backtracking by forming a pol III elongation complex from its component parts using the assembly procedure of Kirieeva et al. (40): 10-nt RNA was annealed to its 51-nt transcribed DNA strand, incubated with pol IIIa, and assembly was completed by the addition of the non-transcribed DNA strand (Fig. S5A). The addition of [$\alpha\text{-}32\text{P}]CTP, UTP, and 3'-OEtGTP generated labeled 16-mer RNA (Fig. 5B, lane 1). Shorter products appeared to result from misincorporation of UTP in place of CTP, as they did not appear when UTP was absent (forming the 13-mer instead, lane 9) or when the 16-mer was formed by the sequential addition of CTP and UTP (data not shown; note that the addition to misincorporated nucleotides is extremely slow (27)). Curiously, while optimizing the conditions for forming the 16-mer RNA-containing elongation complex (by varying the proportions of the two DNA strands and RNA; compare lane 1 with lanes 2–4), we observed that formation of the 16-mer RNA elongation complex required the assembly of the non-transcribed DNA strand (although some extension to nt12 did occur in its absence; data not shown). This may have reflected a role of flanking duplex DNA in stabilizing the initial elongation complex, previously observed for *E. coli* RNA polymerase (41) but not for pol II (40). Inclusion of the C53/C37 subcomplex in these assemblies consistently stimulated the for-
Dual Roles of the Pol III C53/C37 Subcomplex

formation of the 16-mer elongation complex 2–3-fold (Fig. 5B, compare lanes 5–8 with lanes 1–4). Both subunits were required for the stimulation (data not shown), which could not be duplicated by increasing concentrations of the RNA primer or individual DNA strands (lanes 2–4).

The rationale for examining C53-RNA cross-linking with this artificially assembled elongation complex is based on the assumption that backtracking of this complex with its short “transcript” (15-mer RNA with ABU at its 3’ end; Fig. 5, panel A) more than 7 bp would shorten the RNA:DNA hybrid at its upstream end and either block further backtracking or lead to RNA release as a consequence of invasion of the upstream end of the DNA:RNA duplex by the non-transcribed DNA strand (40). Although the 13-mer elongation complex was at least transiently resistant to exposure to 0.5 M NaCl (as evidenced by its ability to be subsequently walked to G16), a significant amount (∼1/2) of the elongation complex dissociated during Sephacryl S300 chromatography (data not shown). Two approaches were taken to eliminate the possible complication of C53 cross-linking to released RNA; 1) C53 addition and UV cross-linking preceded S300 chromatography, and 2) ABU was incorporated subsequent to isolation of the 13-mer RNA elongation complex on Sephacryl S300. The outcome in both cases was similar and is shown for the first method in Fig. 5, panel C. C53 was found to cross-link to ABU at the RNA 3’ end and to co-chromatograph with pol III. The cross-linking efficiency of C53 relative to that of C160 and C128 using either of these approaches was the same (∼2%) as that obtained with a 3’-overhang template when UV irradiation preceded Sephacryl S300 chromatography (Fig. 2B) but was less than the efficiency observed with 3’-overhang templates when ABUMP was incorporated before Sephacryl S300 chromatography and subsequent UV irradiation (e.g. supplemental Fig. S4B; ∼5% C53 cross-linking efficiency relative to C128). In summary, exonuclease III footprinting did not indicate significant backtracking. An artificially assembled elongation complex that should place limits on the extent of backtracking also did not eliminate C53 cross-linking to the 3’ end of the nascent transcript. These findings argue strongly against the supposition that C53 proximity to the nascent RNA 3’ end requires and entirely reflects extensive backtracking of the pol III elongation complex. However, we consistently observed increased C53 cross-linking when NTP substrates were removed before UV irradiation. This observation is compatible with the notion that backtracking (perhaps occurring in a time-dependent process in the absence of NTPs) provides additional sites of C53 proximity to the RNA 3’ end.

The ability of C53 and C37 to stimulate formation of the elongation complex (Fig. 5) prompted an examination of a possible role for these proteins in initiation factor-dependent formation of open promoter complexes. For this purpose we chose the well studied TFIIIB- and TFIIIC-requiring SUP4 tRNA gene and KMnO4 footprinting (Fig. 6), which reveals strand-separated T residues in the open promoter. The addition of wild type pol III to the TFIIIB-TFIIIC-SUP4 DNA complex generated reactivity of non-transcribed strand T residues between bp −2 and −9 (relative to the start site as +1; lane 1) as previously shown (25). At the temperature of this experiment (25 °C), the pol III promoter complex is in dynamic balance between open, partially, and fully closed states (25). The addition of ATP, CTP, UTP, and 3’-OMeGTP to allow formation of an arrested elongation complex at bp +18 shifted the location of reactive T residues to bp +6 to +16 (lane 7); residual KMnO4 reactivity between bp −9 and −2 reflected a minor fraction of elongation-incompetent pol III (36). For initiation complexes assembled with pol IIIΔ, promoter opening was barely detectable above background (lane 3; i.e. shifting the steady state levels of open and closed complexes toward the closed complex), although the formation of arrested elongation complexes was readily apparent (lane 8). The addition of C53, C37, and C11 substantially increased promoter opening (lane 5) and formation of arrested elongation complexes (lane 10), whereas C53 and C37 alone were almost (≥60%) as effectively stimulatory (lanes 4 and 9). These results clearly indicate that the presence of C53 and C37 facilitates promoter opening.

TFIIIB recruits pol III to the start site of transcription; its Brf1 and Bdp1 subunits also participate in subsequent steps of forming the initiation-ready open promoter complex; Bdp1 in nucleating promoter opening at the upstream end of the transcription bubble and Brf1 in propagating DNA strand separation to melt the start site (16, 42, 43). Brf1 lacking its N-terminal zinc ribbon motif (NΔ68) or Bdp1 with small internal deletions covering a 67-amino acid segment (e.g. Δ355–372) generate TFIIIB-pol III-DNA complexes that fail to open on relaxed (linear) DNA templates. Combining both mutant subunits in TFIIIB leads to a loss of ability to transcribe supercoiled DNA (16). We examined whether pol III and pol IIIΔ would respond differently to these TFIIIB defects in vitro; that is, whether synthetic phenotypes would materialize in combinations of pol IIIΔ and defective TFIIIB. For the analysis, we used plasmid
DUAL ROLES OF THE POL III C53/C37 SUBCOMPLEX

A Functional Relationship of the C53/C37 Subcomplex to TFIIIF—On the basis of limited sequence similarity and predicted secondary structure, it has been suggested that the C53/C37 subcomplex may be related to the pol II elongation factor TFIIH; the specifics of the C53/C37:TFIIF relationship was not provided (13, 44), but a potential similarity between the N-terminal zinc ribbon region. The C11 subunit also appears to contribute in this process.

DISCUSSION

A Functional Relationship of the C53/C37 Subcomplex to TFIIIF—On the basis of limited sequence similarity and predicted secondary structure, it has been suggested that the C53/C37 subcomplex may be related to the pol II elongation factor TFIIH; the specifics of the C53/C37:TFIIF relationship was not provided (13, 44), but a potential similarity between the N-terminal zinc ribbon region. The C11 subunit also appears to contribute in this process.

FIGURE 7. Pol IIIΔ places additional demands on TFIIIB for initiation of transcription. A, the TATA box of the SNR6 (U6 small nuclear RNA)-derived plasmid pY185S permits ambidirectional binding of TFIIIB and generates divergent transcription (see supplemental Fig. S7). Transcription of supercoiled plasmid DNA directed by TFIIIB assembled with wild type (wt, reference type) and mutant Brf1 and Bdp1 is shown. The Brf1 mutation deleting its N-terminal zinc binding domain (residues 1–68; NΔ) was introduced into the background of Brf1(Δ366–408) (rt); removing the latter non-conserved 43-residue segment increases the activity of Brf1 in vitro. The Bdp1 mutation removing residues 355–372 (intΔ) was introduced into the wild type background. TFIIIB-promoter complexes were formed before the addition of wild type pol III or pol IIIΔ, with or without 25 ng C53, C37, and C11 followed in turn by the addition of NTPs for 30 min of multiple-round transcription. Leftward (U6L) and rightward (U6R) transcripts are identified at the left side. Asterisks between lanes 6 and 7 point to the increased length of pol IIIΔ transcripts reflecting defective termination that is corrected by C53 and C37 (lanes 8 and 9). TFIIIB assembled with NAΔ88Brf1 is essentially inactive for transcription by pol IIIΔ (lane 7). B, shown is the quantitative presentation of panel A (with U6L and U6R transcripts in lanes 2 and 3 separately normalized to their counterparts in lane 1 and transcripts in lanes 6–9 separately normalized to lane 5) showing partial restoration of activity to pol IIIΔ by C53 and C37, further improved by C11 (less than half of the sample analyzed in panel A, lane 1, was recovered, but this has been corrected relative to its recovery marker in panel B).

(superciled) DNA containing a variant of the U6 small nuclear RNA gene (SNR6), which allows TFIIIC-independent, ambidirectional binding of TFIIIB to its strong and nearly symmetric TATA box, and monitored the resulting divergent transcription (U6L and U6R in Fig. 7; schematically diagrammed in supplemental Fig. S7). Lanes 1–4 of Fig. 7A recapitulate the properties of wild type pol III (16), including the substantial transcriptional activity of TFIIIB assembled with the individual mutant Brf1 and Bdp1 subunits and inactivity of TFIIIB containing both mutant subunits. The U6R and U6L transcription units contain T10 terminators, but the termination defect of pol IIIΔ is clearly evident by the shift to the start site-distal end of the T10 stretch (Fig. 7A, compare lanes 5 and 6 with lanes 1 and 2). The effect of the Bdp1 and Brf1 mutations on transcription with wild type pol III and pol IIIΔ is quantified (relative to a sample recovery marker, not shown) and normalized to transcription by each polymerase with wild type TFIIIB in panel B. Although no synthetic effect of these mutations was seen for the pol IIIΔ/Bdp1(Δ355–372) combination (panel B, compare lanes 2 and 6), the combination of pol IIIΔ with TFIIIB containing Brf1(NAΔ68) yielded no U6L transcription and only trace levels of U6R transcripts (compare lanes 3 and 7). The addition of C53, C37, and C11 substantially restored transcription (compare lanes 8 and 3), whereas C53 and C37 alone partially did so (compare lane 9 with lane 7). The addition of C53, C37, and C11 did not stimulate pol IIIΔ transcription when assembled with wild type TFIIIB (data not shown). C11 does not appear to function by stabilizing the interaction of C53/C37 with pol IIIΔ, as an ~4-fold increase in C53 and C37 concentration did not further restore transcription with TFIIIB containing Brf1 NAΔ68 (supplemental Fig. S7). No restoration of transcription was achieved by C11 alone (supplemental Fig. S7) nor by combinations of C53 or C37 with C11 (data not shown).

These results indicate that the C53/C37 subcomplex is required for downstream propagation of the transcription bubble during formation of the open promoter complex on supercoiled DNA templates in conjunction with TFIIIB lacking the Brf1 zinc ribbon region. The C11 subunit also appears to contribute in this process.

FIGURE 7. Pol IIIΔ places additional demands on TFIIIB for initiation of transcription. A, the TATA box of the SNR6 (U6 small nuclear RNA)-derived plasmid pY185S permits ambidirectional binding of TFIIIB and generates divergent transcription (see supplemental Fig. S7). Transcription of supercoiled plasmid DNA directed by TFIIIB assembled with wild type (wt, reference type) and mutant Brf1 and Bdp1 is shown. The Brf1 mutation deleting its N-terminal zinc binding domain (residues 1–68; NΔ) was introduced into the background of Brf1(Δ366–408) (rt); removing the latter non-conserved 43-residue segment increases the activity of Brf1 in vitro. The Bdp1 mutation removing residues 355–372 (intΔ) was introduced into the wild type background. TFIIIB-promoter complexes were formed before the addition of wild type pol III or pol IIIΔ, with or without 25 ng C53, C37, and C11 followed in turn by the addition of NTPs for 30 min of multiple-round transcription. Leftward (U6L) and rightward (U6R) transcripts are identified at the left side. Asterisks between lanes 6 and 7 point to the increased length of pol IIIΔ transcripts reflecting defective termination that is corrected by C53 and C37 (lanes 8 and 9). TFIIIB assembled with NAΔ88Brf1 is essentially inactive for transcription by pol IIIΔ (lane 7). B, shown is the quantitative presentation of panel A (with U6L and U6R transcripts in lanes 2 and 3 separately normalized to their counterparts in lane 1 and transcripts in lanes 6–9 separately normalized to lane 5) showing partial restoration of activity to pol IIIΔ by C53 and C37, further improved by C11 (less than half of the sample analyzed in panel A, lane 1, was recovered, but this has been corrected relative to its recovery marker in panel B).
located near the catalytic center of the pol II initiation complex (47–50). 2) The N-terminal zinc ribbon regions of Brf1, TFIIB, and archaeal TFB probably plays similar post-polymerase recruitment roles in stabilizing the open complex and the initially forming short RNA:DNA hybrid through interaction with their respective B finger motifs (16, 47, 51–53). Just as the C53/C37 subcomplex is required for transcription of a supercoiled DNA template when the zinc ribbon region of Brf1 is removed (Fig. 7), TFIIF can compensate for TFIIB B-finger mutations that reduce or eliminate transcription in a minimal pol II transcription system consisting of TBP, TFIIB, pol II, and a supercoiled DNA template (54). 3) The C53/C37 subcomplex stimulates the artificial assembly of a pol III elongation complex from its RNA and DNA components (Fig. 5), and TFIIF exerts a comparable effect in stabilizing the incorporation of a 5 nt RNA into a pol II heteroduplex DNA complex containing a 10-bp bubble (55). In addition to these functional similarities, TFIIF and the C53/C37 subcomplex appear to attach to similar sites on the second largest subunits of their respective RNA polymerases, the Rpb2/C128 lobe domains above the DNA binding cleft (11, 48), and the stability of these interactions is also dependent on attachment of the respective paralogue Rpb9/C11 subunits to the lobe domain (7, 8, 56).

The proximity of C53 to the pol III catalytic center suggests that the C53/C37 subcomplex may participate directly in open complex formation and also in potentiating transcriptional pausing and termination. With respect to open complex formation, C53 may interact directly with the transcribed DNA strand, to which it cross-links in the elongation complex (Fig. 4), and thus, stabilize localized DNA strand separation. It has been suggested that stimulation of pausing is coupled to termination because increasing the dwell time of the elongation complex at the terminator increases the probability that the elongation complex will disassemble (8). The simple recognition element for terminating pol III transcription consists of a run of five or more adjacent T residues in the non-transcribed strand (57, 58), whereas the strengths of intrinsic pause sites for pol III strongly correlate with increasing numbers of U residues at the nascent transcript 3’ end (59). Sites of pausing and termination are also sites of enhanced C11 subunit-mediated exoribonucleolytic cleavage (60); both pausing and cleavage are likely to arise from fraying of the RNA 3’ end due to the instability of the RNA:DNA hybrid (61); all of the termination-deficient mutations of Shaaban et al. (62) can be mapped to the lobe and fork domains of C128. Eight mutations in the lobe are clustered in the N-terminal half of helix α9 and the αα-α9 loop (corresponding to Rpb2 residues 338–354). This C128 segment lies proximal to (within ~25 Å) of the N-terminal zinc ribbon domain of C11 but inclined toward the DNA binding cleft and may constitute a C53/C37 binding site. More than one-half of the fork-domain mutations cluster within the 22-residue segment of C128 that contains fork loop 2 at its center (corresponding to Rpb2 residues 496–517). In the structure of pol II, the mobile fork loop 2 (Rpb2 residues 501–511) is positioned to define the downstream edge of the transcription bubble in the elongation complex by blocking access of the non-transcribed DNA strand at register +3 to the catalytic site as duplex DNA (63, 64). Fork loop 2 moves in concert with DNA at register +3 and maintains its interaction with it, suggesting a role in the unwinding of downstream DNA (65). It has also been proposed that this downstream DNA interaction may contribute to the stability of the paused elongation complex (66).

It has recently been proposed that Rpb9 prevents misincorporation of noncognate NTPs from its lobe-surface location through a direct interaction with the Rpb1 trigger loop that delays closure of the latter (the trigger loop), which in turn affects the sequestration and proper positioning of the incoming NTP for catalysis (67). We suggest that, in a similar manner, the C53/C37 subcomplex may affect elongation and termination through direct or allosteric interactions that alter the mobility of fork loop 2.

*Is the C11 Subunit Located at the Pol III Catalytic Center?—*

The sequence similarity of the C11 and TFIIS C-terminal domains and their similar function of promoting exoribonucleolytic proofreading suggested the possibility that the tip of the C11 β finger might also be located in proximity to the 3’ end of the nascent transcript. RNA-protein cross-linking analysis was originally undertaken to clarify whether this is the case. However, the C11-RNA cross-linking that we have observed appears not to be associated with the pol III elongation complex but, rather, results from nonspecific binding of C11 to low levels of released RNA (Figs. 2A and 3). Our attempts to demonstrate a residue of specific RNA cross-linking (i.e. in the elongation complex) above this nonspecific background proved unsuccessful (data not shown). This negative outcome does not eliminate the possibility that the C11 β finger lies proximal to the RNA 3’ end of the pol III elongation complex. For example, steric occlusion (by C128, for example) may have prevented access of the RNA-attached photoactive function to C11. The amino acid selectivity of cross-linking by aryl azides is an additional consideration; for example, substituting the azide of 3ABU with diazirine revealed the proximity of additional pol III subunits to DNA at certain locations of a pol III promoter complex (68).

Proximity of the C53 Subunit to the Pol III Catalytic Center—

Although C53 also binds and cross-links to free RNA containing ABU at its 3’ end (Fig. 3), our evidence indicates that C53-RNA cross-linking occurs within the elongation complex at or near its catalytic center: 1) C53 cross-linked to ABU at the 3’ end of the nascent transcript co-chromatographed perfectly.
with the pol III elongation complex (Figs. 2B and 5C), 2) isolation of pol IIIΔ elongation complexes (under conditions stringently removing released RNA) before C53/C37 addition and UV irradiation also resulted in C53-RNA cross-linking (supplemental Figs. S3 and S4B) and 3) C53 was seen to cross-link to the transcribed DNA strand at register −1/−2 under conditions that required the formation of a stable elongation complex (Fig. 4). These observations are also consistent with finding that pol III lacking the C53/C37 subcomplex has a promoter-opening defect (Figs. 6 and 7) and with proximity of C37 to the nontranscribed strand of the elongation complex at register −2/−3 (36).

Heparin Sensitivity of C53-DNA Cross-linking—Observations on the interaction of C53 with the polyanion heparin somewhat complicate this picture and require further comment. Heparin, a single-stranded-DNA/RNA mimic, has been somewhat complicated this picture and require further comments and advice from the inception and throughout the course of this work and for sustained editorial help, David J. Stillman and Lorraine Pillus, and Suresh Subramani for generously providing materials.

Acknowledgments—We thank E. P. Geiduschek for many discussions and advice from the outset of the manuscript. We thank David J. Stillman, Lorraine Pillus, and Suresh Subramani for generously providing materials.

REFERENCES

1. Dieci, G., Fiorino, G., Castelnuovo, M., Teichmann, M., and Pagano, A. (2007) Trends Genet. 23, 614–622
2. Schramm, L., and Hernandez, N. (2002) Genes Dev. 16, 2593–2620
3. Geiduschek, E. P., and Kassavetis, G. A. (2001) J. Mol. Biol. 310, 1–26
4. Kassavetis, G. A., Braun, B. R., Nguyen, L. H., and Geiduschek, E. P. (1990) Cell 60, 235–245
5. Soragni, E., and Kassavetis, G. A. (2008) J. Biol. Chem. 283, 26568–26576
6. Werner, M., Chaussvert, N., Willis, I. M., and Sentenac, A. (1993) J. Biol. Chem. 268, 20721–20724
7. Chédin, S., Riva, M., Schultz, P., Sentenac, A., and Carles, C. (1998) Genes Dev. 12, 3857–3871
8. Landrieux, E., Alic, N., Ducrot, C., Acker, J., Riva, M., and Carles, C. (2006) EMBO J. 25, 118–128
9. Whitehall, S. K., Bardeleben, C., and Kassavetis, G. A. (1994) J. Biol. Chem. 269, 2299–2306
10. Kettenberger, H., Armache, K. J., and Cramer, P. (2003) Cell 114, 347–357
11. Fernández-Tornero, C., Böttcher, B., Riva, M., Carles, C., Steuerwald, U., Ruigrok, R. W., Sentenac, A., Müller, C. W., and Schoenh, G. (2007) Mol. Cell 25, 813–823
12. Cramer, P., Bushnell, D. A., and Kornberg, R. D. (2001) Science 292, 1863–1876
13. Kuhn, C. D., Geiger, S. R., Bauml, S., Gartmann, M., Gerber, J., Jenneweck, S., Mielke, T., Tschochner, H., Beckmann, R., and Cramer, P. (2007) Cell 131, 1260–1272
14. Bartholomew, B., Tinker, R. L., Kassavetis, G. A., and Geiduschek, E. P. (1995) Methods Enzymol. 262, 476–494
15. Joazeiro, C. A., Kassavetis, G. A., and Geiduschek, E. P. (1996) Genes Dev. 10, 725–739
16. Kassavetis, G. A., Letts, G. A., and Geiduschek, E. P. (2001) EMBO J. 20, 2823–2834
17. Goldstein, A. L., and McCusker, J. H. (1999) Yeast 15, 1541–1553
18. Yoth, W. P., Jiang, Y. W., and Stillman, D. J. (2003) Yeast 20, 985–993
19. Guthrie, C., and Fink, G. R. (eds) (1991) Guide to Yeast Genetics and Molecular Biology, Academic Press, Inc., San Diego, CA.
20. Sikorski, R. S., and Hieter, P. (1989) Genetics 122, 19–27
21. Nirgo, J., Sikorski, R., Reed, S. I., and Vogelstein, B. (1992) Mol. Cell. Biol. 12, 1357–1365
22. Kassavetis, G. A., Soragni, E., Driscoll, R., and Geiduschek, E. P. (2005) Proc. Natl. Acad. Sci. U.S.A. 102, 15406–15411
23. Kassavetis, G. A., Riggs, D. L., Negri, R., Nguyen, L. H., and Geiduschek, E. P. (1989) Mol. Cell. Biol. 9, 2551–2566
24. Yieh, L., Kassavetis, G., Geiduschek, E. P., and Sandmeyer, S. B. (2000) J. Biol. Chem. 275, 29800–29807
25. Kassavetis, G. A., Blanco, J. A., Johnson, T. E., and Geiduschek, E. P. (1992) J. Mol. Biol. 226, 47–58
26. Powell, W., Bartholomew, B., and Reines, D. (1996) J. Biol. Chem. 271, 22301–22304
27. Alic, N., Ayoub, N., Landrieux, E., Favre, E., Baudouin-Cornu, P., Riva, M., and Carles, C. (2007) Proc. Natl. Acad. Sci. U.S.A. 104, 10400–10405
28. Bardeleben, C., Kassavetis, G. A., and Geiduschek, E. P. (1994) J. Mol. Biol. 235, 1193–1205
29. Kassavetis, G. A., Zentner, P. G., and Geiduschek, E. P. (1986) J. Biol. Chem. 261, 14256–14265
30. Dieci, G., Herrmann-Le Dennat, S., Lukhtanov, E., Thuriaux, P., Werner, M., and Sentenac, A. (1995) EMBO J. 14, 3766–3776
31. Dedrick, R. L., and Chamberlin, M. J. (1985) Biochemistry 24, 2245–2253
32. Hanna, M. M., Zhang, Y., Reidling, J. C., Thomas, M. J., and Jø, J. (1993) Nucleic Acids Res. 21, 2073–2079
33. Agarwal, K., Baek, K. H., Jeon, C. J., Miyamoto, K., Ueno, A., and Yoon, H. S. (1991) Biochemistry 30, 7842–7851
34. Bartholomew, B., Durkovich, D., Kassavetis, G. A., and Geiduschek, E. P. (1993) Mol. Cell. Biol. 13, 942–952
35. Lannutti, B. J., Persinger, J., and Bartholomew, B. (1996) Biochemistry 35, 9821–9831
36. Bartholomew, B., Braun, B. R., Kassavetis, G. A., and Geiduschek, E. P. (1994) J. Biol. Chem. 269, 18090–18095
37. Jasiak, A. J., Armache, K. J., Martens, B., Jansen, R. P., and Cramer, P. (2006) Mol. Cell 23, 71–81
38. Nudler, E., Goldfarb, A., and Kashlev, M. (1994) Science 265, 793–796
39. Woodwell, C. L., and Burgess, R. R. (2000) Biochemistry 39, 13405–13421
40. Kireeva, M. L., Komissarova, N., Waugh, D. S., and Kashlev, M. (2000) J. Biol. Chem. 275, 6530–6536
41. Sidorenkov, I., Komissarova, N., and Kashlev, M. (1998) Mol. Cell 2, 55–64
42. Kassavetis, G. A., Kumar, A., Letts, G. A., and Geiduschek, E. P. (1998) Proc. Natl. Acad. Sci. U.S.A. 95, 9196–9201
43. Kassavetis, G. A., Grove, A., and Geiduschek, E. P. (2002) EMBO J. 21, 5508–5515
44. Cramer, P., Armache, K. J., Bauml, S., Benkert, S., Buclak, F., Buchen, C., Damsma, G. E., Dengl, S., Geiger, S. R., Jasiak, A. J., Jawhari, A., Jennebach, S., Kamenski, T., Kettenberger, H., Kuhn, C. D., Lehmam, E., Leike, K., Sydow, J. F., and Vannini, A. (2008) Annu. Rev. Biophys. 37, 337–352
45. Söding, J., Biegert, A., and Lupas, A. N. (2005) Nucleic Acids Res. 33, W244–W248
Dual Roles of the Pol III C53/C37 Subcomplex

46. Zhang, C., Zobeck, K. L., and Burton, Z. F. (2005) Mol. Cell. Biol. 25, 3583–3595
47. Chen, H. T., and Hahn, S. (2004) Cell 119, 169–180
48. Chen, H. T., Warfield, L., and Hahn, S. (2007) Nat. Struct. Mol. Biol. 14, 696–703
49. Freire-Picos, M. A., Krishnamurthy, S., Sun, Z. W., and Hampsey, M. (2005) Nucleic Acids Res. 33, 5045–5052
50. Chen, B. S., Mandal, S. S., and Hampsey, M. (2004) Biochemistry 43, 12741–12749
51. Bushnell, D. A., Westover, K. D., Davis, R. E., and Kornberg, R. D. (2004) Science 303, 983–988
52. Werner, F., and Weinzierl, R. O. (2005) Mol. Cell. Biol. 25, 8344–8355
53. Micorescu, M., Grünberg, S., Franke, A., Cramer, P., Thomm, M., and Bartlett, M. (2008) J. Bacteriol. 190, 157–167
54. Thompson, N. E., Glaser, B. T., Foley, K. M., Burton, Z. F., and Burgess, R. R. (2009) J. Biol. Chem. 284, 24754–24766
55. Khaperskyy, D. A., Ammerman, M. L., Majovski, R. C., and Ponticelli, A. S. (2008) Mol. Cell. Biol. 28, 3757–3766
56. Ziegler, L. M., Khaperskyy, D. A., Ammerman, M. L., and Ponticelli, A. S. (2003) J. Biol. Chem. 278, 48950–48956
57. Campbell, F. E., Jr., and Setzer, D. R. (1992) Mol. Cell. Biol. 12, 2260–2272
58. Hamada, M., Sakulich, A. L., Koduru, S. B., and Marais, R. J. (2000) J. Biol. Chem. 275, 29076–29081
59. Matsuzaki, H., Kassavetis, G. A., and Geiduschek, E. P. (1994) J. Mol. Biol. 235, 1173–1192
60. Bobkova, E. V., and Hall, B. D. (1997) J. Biol. Chem. 272, 22832–22839
61. Shaaban, S. A., Bobkova, E. V., Chudzik, D. M., and Hall, B. D. (1996) Mol. Cell. Biol. 16, 6468–6476
62. Shaaban, S. A., Krupp, B. M., and Hall, B. D. (1995) Mol. Cell. Biol. 15, 1467–1478
63. Gnatt, A. L., Cramer, P., Fu, J., Bushnell, D. A., and Kornberg, R. D. (2001) Science 292, 1876–1882
64. Kettenberger, H., Armache, K. J., and Cramer, P. (2004) Mol. Cell 16, 955–965
65. Wang, D., Bushnell, D. A., Westover, K. D., Kaplan, C. D., and Kornberg, R. D. (2006) Cell 127, 941–954
66. Sydow, J. F., Brueckner, F., Cheung, A. C., Damsma, G. E., Dengi, S., Lehmann, E., Vassylev, D., and Cramer, P. (2009) Mol. Cell 34, 710–721
67. Walmacq, C., Kireeva, M. L., Irvin, J., Nedialkov, Y., Lubkowska, L., Malagon, F., Strathern, J. N., and Kashlev, M. (2009) J. Biol. Chem. 284, 19601–19612
68. Tate, J. J., Persinger, J., and Bartholomew, B. (1998) Nucleic Acids Res. 26, 1421–1426
69. Kassavetis, G. A., Nguyen, S. T., Kobayashi, R., Kumar, A., Geiduschek, E. P., and Pisano, M. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 9786–9790