THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 37, Issue of September 10, pp. 26550–26556, 1999
Printed in U.S.A.
© 1999 by The American Society for Biochemistry and Molecular Biology, Inc.

Abortive Initiation by *Saccharomyces cerevisiae* RNA Polymerase III*

(Received for publication, May 11, 1999)

Purnima Bhargava‡§ and George A. Kassavetis§

From the Department of Biology and Center for Molecular Genetics, University of California, San Diego, La Jolla, California 92093-0634

Promoter escape can be rate-limiting for transcription by bacterial RNA polymerases and RNA polymerase II of higher eukaryotes. Formation of a productive elongation complex requires disengagement of RNA polymerase from promoter-bound eukaryotic transcription factors or bacterial sigma factors. RNA polymerase III (pol III) stably associates with the TFIIIB-DNA complex even in the absence of localized DNA unwinding associated with the open promoter complex. To explore the role that release of pol III from the TFIIIB-DNA complex plays in limiting the overall rate of transcription, we have examined the early steps of RNA synthesis. We find that, on average, only three rounds of abortive initiation precede the formation of each elongation complex and that nearly all pol III molecules escape the abortive initiation phase of transcription without significant pausing or arrest. However, when elongation is limited to 5 nucleotides, the intrinsic exoribonuclease activity of pol III cleaves 5-mer RNA at a rate considerably faster than product release or reinitiation. This cleavage also occurs in the normal process of forming a productive elongation complex. The possible role of nucleolytic retraction in disengaging pol III from TFIIIB is discussed.

RNA polymerase III (pol III)[1] transcribes genes encoding small RNAs involved in translation and RNA processing (*e.g.* tRNAs, 5 S ribosomal RNA, U6 small nuclear RNA). Pol III from *Saccharomyces cerevisiae* is recruited to the transcriptional start sites of these genes by its central transcription factor TFIIIB, which is composed of three subunits: TBP, Brf, and B″. TFIIIB is assembled upstream of the start site of transcription by its assembly factor TFIIIC, whose binding site lies in the transcribed DNA sequence. Once assembled, TFIIIB forms a highly stable complex on DNA and alone suffices for recruiting pol III for multiple rounds of transcription (for recent reviews, see Refs. 1 and 2).

Much of the analysis of the *S. cerevisiae* pol III transcription system has centered on the assembly, roles, and properties of its transcription factors (reviewed as cited above) and to a lesser extent on the dynamics of RNA chain elongation and termination (3–12). Only recently has emphasis been placed on the steps that occur between the recruitment of pol III into the complex and the formation of a productive, stable elongation complex. What is known is reminiscent of the pathway that bacterial RNA polymerases follow in forming a productive elongation complex. Pol III assembles onto a TFIIIB-DNA complex at the start site of transcription at 0 °C to form a relatively stable closed complex (*i.e.* without melting DNA) (13). Closed promoter complexes specified by σ^{54}-containing *Escherichia coli* RNA polymerase holoenzyme are likewise stable, in contrast to the generally unstable closed promoter complexes specified by the σ^{70}-holoenzyme (14, 15). DNA melting by pol III is a temperature-dependent process that initially occurs upstream of the start site of transcription and then extends downstream (13), as has been noted subsequently for open complex formation formed by bacterial RNA polymerases (*e.g.* Refs. 16–18). Recent experiments point to an active participation of TFIIIB in open complex formation by pol III (19, 20).

Following open complex formation, both bacterial RNA polymerases and RNA polymerase II (pol II) of higher eukaryotes enter an abortive initiation phase of templated reiterative synthesis, yielding short ribooligonucleotide products, usually ranging between 2 and 10 nt in length (21–29). In the case of the bacterial RNA polymerase, the number of abortive products generated per productive elongation complex can be high (exceeding 50; Refs. 21 and 24) The relative frequency of abortive to productive initiation is influenced both by the promoter and by the initially transcribed sequence and can be rate-limiting for gene expression (30, 31). The transition from abortive initiation to productive elongation is made irreversible by the release of σ^{70} and movement of RNA polymerase away from the promoter (22, 23, 25, 32).

Pol II also undergoes a transition from an abortive phase to a productive complex after synthesis of a 9-nt transcript and concurrent reclosure of the transcription bubble upstream of the start site of transcription (28). Both initiation and escape from the abortive phase at the adenovirus major late promoter are facilitated by TFIIE and TFIIH (29, 33). In the absence of TFIIH, pol II arrests 10–17 nt downstream of the start site of transcription (29, 34). The presence of TFIIE, TFIIH, and ATP greatly diminishes this arrest and also diminishes the production of short abortive transcripts. Both the elongation factor elongin and the VP16 transcriptional activator further diminish this arrest (29, 35).

Pol II is also subject to an additional promoter-proximal block to transcript elongation that is likely to be mechanistically distinctive. RNA chain elongation arrests within the first 50 bp of many pol II transcription units in the absence of gene-specific activators (Refs. 36–38; for reviews, see Refs. 39 and 40), which may function to facilitate the mediator-elongator exchange phase of the transcription cycle (41, 42).

The activity of pol III can also be limited at this stage of

* This work was supported by NIGMS, National Institutes of Health, Grant GM 18386. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of an Overseas Fellowship from the Department of Biotechnology, Government of India. Present Address: Center for Cellular and Molecular Biology, Uppal Rd., Tarnaka, Hyderabad 500007, India.

§ To whom correspondence may be addressed: Dept. of Biology and Center for Molecular Genetics, University of California, San Diego, 9500 Gilman Dr., La Jolla, CA 92093-0634. Tel.: 619-534-2451; Fax: 619-534-7073; E-mail: gak@ucsd.edu.; purnima@ccmb.ap.nic.in.

[1] The abbreviations used are: pol III, polymerase III; pol II, polymerase II; nt, nucleotide(s); bp, base pair(s); 3′O-MeGTP, 3′-*O*-methyl-GTP.

This paper is available on line at http://www.jbc.org

abortive initiation; a defect in the transition to productive transcription of poly(dA-dT):poly(dA-dT) by a pol III C160 subunit mutant has been noted (9). The production of repetitive sequence RNA by "slippage" at low concentrations of initiating nucleotides also implies the existence of an abortive phase of transcription on natural pol III templates (5, 43). We have been interested in whether the escape from abortive initiation might significantly limit the overall rate of pol III transcription. This appears plausible; pol III forms a stable complex with TFIIIB on DNA even in the absence of promoter opening, and promoter clearance is likely to necessitate the breaking of this protein-protein contact. The affinity of bacterial RNA polymerase for the promoter and of the core enzyme for σ can also govern the duration of the abortive phase of initiation and the sizes of abortive transcripts (44, 45). The rapidity (generally less than 3–5 s) with which pol III completes the elongation phase on its short (generally less than 110-bp) transcription units also suggests that the abortive phase of transcription might be rate-limiting.

The stability of the pol III-TFIIIB-DNA complex suggests that a signature of pol III breaking away from TFIIIB might be discerned among the abortive transcripts, either through extended repetitive abortive initiation (as observed with bacterial RNA polymerase) or through start site-proximal pausing or arrest (as observed for pol II). In the experiments that are reported here, we have examined abortive initiation and post-initiation steps leading to the formation of a stable, productive elongation complex. We find that nearly all pol III-promoter complexes that are capable of undergoing abortive initiation are also capable of generating a productive elongation complex. The number of abortive initiation events on the pathway to formation of a productive elongation complex is small; moreover, no hold-up points in extending an RNA chain from 5 to 18 nt are detected. Curiously, however, we observe a relatively efficient process of transcription start-proximal hydrolytic retraction of nascent RNA chains; under conditions limiting transcription to the formation of the 5-nt transcript, the latter is rapidly cleaved by the intrinsic ribonuclease activity of pol III.

MATERIALS AND METHODS

TFIIIC, pol III, recombinant TBP, Brf, and B″ were purified and assayed as described previously or cited (46). The DNA template for transcription, pTA-30 (47), contains a 6-bp TATA box placed within a GC-rich sequence context 30 bp upstream of the transcription start of the wild type *SUP4* tRNA gene (Fig. 1*A*). Oligoribonucleotides ApApC, ApApCpA, and ApApCpApA, dinucleoside monophosphates, ribonuclease A, ribonuclease U2, shrimp alkaline phosphatase, calf intestinal alkaline phosphatase, [α-^{32}P]ATP, [α-^{32}P]UTP, [α-^{32}P]CTP, unlabeled ribonucleotides, and 3′-*O*-methyl-GTP (3′O-MeGTP) were purchased.

Transcription—Transcription complexes were formed for 1 h at 21 °C with 50 fmol of pTA-30, 100 fmol of TBP, 75 fmol of B″, 30 fmol of Brf, 37 fmol of TFIIIC, and 8 fmol of pol III in 8 μl of a reaction mixture containing 40 mM Tris-Cl (pH 8.0), 7 mM $MgCl_2$, 3 mM dithiothreitol, 100 μg/ml bovine serum albumin, 12% (v/v) glycerol, and 120 mM NaCl. Transcription was initiated by adding 2 μl of a nucleotide mixture providing 25 μM [α-^{32}P]ATP (30 cpm/fmol), 25 μM CTP, 25 μM UTP, and 100 μM 3′O-MeGTP, as indicated in the figures. Reactions were terminated after 0.5–30 min at 21 °C by plunging reaction tubes into a 100 °C bath for 3 min. Samples were then cooled to room temperature, and 1 unit of shrimp alkaline phosphatase was added for 15 min at 37 °C to release radioactive orthophosphate from [α-^{32}P]ATP and from the 5′-ends of transcripts. Reaction mixtures were then transferred to tubes containing a quantity of (dry) urea providing 6 M final concentration, heated at 100 °C for 3 min, resolved by electrophoresis on 20% polyacrylamide (22:3 acrylamide:bisacrylamide) gel containing 8 M urea, and quantified by phosphor image analysis.

Stable ternary transcription complexes were isolated by chromatography on Sepharose CL2B (48). For these experiments, reaction mixtures were formed as above, but on a 5-fold larger scale, and RNA synthesis was terminated after 15 min at 21 °C by adding EDTA to 10 mM. The reaction mixture was then chromatographed on Sepharose CL2B in transcription buffer lacking $MgCl_2$ but containing 0.01% Tween 20, 0.2 mM EDTA, and 5% (v/v) glycerol. Plasmid DNA-containing fractions in the excluded volume were pooled; where indicated, $MgCl_2$ to 7 mM; ATP, CTP, and UTP to 25 μM; and 3′O-MeGTP to 100 μM were added for 15 min at 21 °C. Reactions were terminated by heating to 100 °C followed by digestion with alkaline phosphatase and electrophoretic analysis, as described above.

Identification of Abortive Initiation Products—Short oligoribonucleotides generated by pol III in the presence of ATP, ATP plus CTP, or ATP plus CTP plus UTP after 30 min of synthesis were identified by passive elution of radioactive material from slices ("bands") excised from electrophoresis gels (Fig. 1*B*) into 200 μl of 100 mM NH_4 acetate, 1 mM EDTA. The eluted oligonucleotides were treated with ribonuclease A, ribonuclease U2, or alkaline phosphatase and analyzed by chromatography on polyethyleneimine thin layers with 0.16, 0.3, and 0.5 M LiCl as solvent (49) in conjunction with appropriate unlabeled mono-, di-, tri-, tetra-, and pentanucleotide markers.

An example of the analysis is shown in Fig. 1*C*. Band I of Fig. 1*B* (from the ATP-only reaction of Fig. 2*A*) was identified as ApA, since it was synthesized in reactions containing only ATP, the product co-migrated on thin layers with ApA, it failed to be cleaved by ribonuclease U2 (which cleaves ApApA, but ApA cleavage is fairly resistant; Ref. 50), and its migration on thin layers was unaffected by alkaline phosphatase. Band II from the ATP plus CTP reaction was also identified as ApA by the same criteria. In comparable ways, band III was identified as CpA, and band V was identified as ApApCpApA. Band III co-migrated with CpA and not ApC on thin layers; it was cleaved by RNase A, generating ^{32}P-labeled 3′-CMP (Fig. 1*C*). Band V co-migrated with ApApCpApA on thin layers. Band IV was identified as predominately ApApC but contained trace amounts of what most probably is ApApCpA (but conceivably could be ApCpApA). The major and trace products that were resolved on thin layer chromatography co-migrated with ApApC and ApApCpA, respectively; the major product was not cleaved by RNase A or affected by alkaline phosphatase but was cleaved by RNase U2, generating a product co-migrating with ApAp on thin layers. (The latter product was synthesized by pol III with ApA and [α-^{32}P]CTP on the *SUP4* (pTA-30) template, followed by cleavage with RNase U2.) Bands VI and X (presumed to be due to incomplete dephosphorylation prior to gel electrophoresis) were not consistently observed; their contents migrated on polyethyleneimine thin layers ahead of the dinucleoside monophosphates and were not further identified. Bands VII and VIII were identified as ApA and CpA, respectively, by the same criteria applied to bands II and III. Although the band IX product migrates on polyacrylamide gels with band IV, it was found to contain only trace ApApC (as evidenced by its co-migration with an ApApC marker on polyethyleneimine thin layers) and no detectable ApApCpA. Most of this material appears to be UpA; it co-migrated with UpA on thin layers, was cleaved by RNase A generating ^{32}P-nucleoside monophosphate, and was not cleaved by alkaline phosphatase or RNase U2.

RESULTS

It has been shown previously that pol III forms a highly stable ternary elongation complex with a 17-nt nascent RNA chain when it initiates transcription on the *SUP4* tRNA gene with a nucleotide mixture lacking GTP. Elongation of the nascent RNA resumes promptly and quantitatively upon subsequent provision of GTP (51, 52). However, it is not known whether there is a kinetically significant phase of abortive initiation prior to the formation of this stable elongation complex or whether there are sites of pausing on the way to forming the 17-mer. In a crude cell-free extract, pol III initiates transcription exclusively at bp +1 in the sequence shown in Fig. 1*A*. However, with highly purified components derived entirely from yeast or with purified pol III, TFIIIC, and recombinant TFIIIB, somewhat heterogeneous placement of TFIIIB by TFIIIC onto the AT-rich sequence upstream of the transcriptional start of the *SUP4* tRNA gene generates additional start sites at bp +4 and +8 (Refs. 47 and 53; data not shown). In order to specify the site of transcriptional initiation more precisely, plasmid pTA-30 was chosen as template. Its 6-bp TATA box in a GC-rich sequence context upstream of the *SUP4* tRNA gene has been designed to retain TFIIIC dependence for TFIIIB assembly while forcing a unique positioning of TFIIIB that specifies initiation at bp +1 (47).

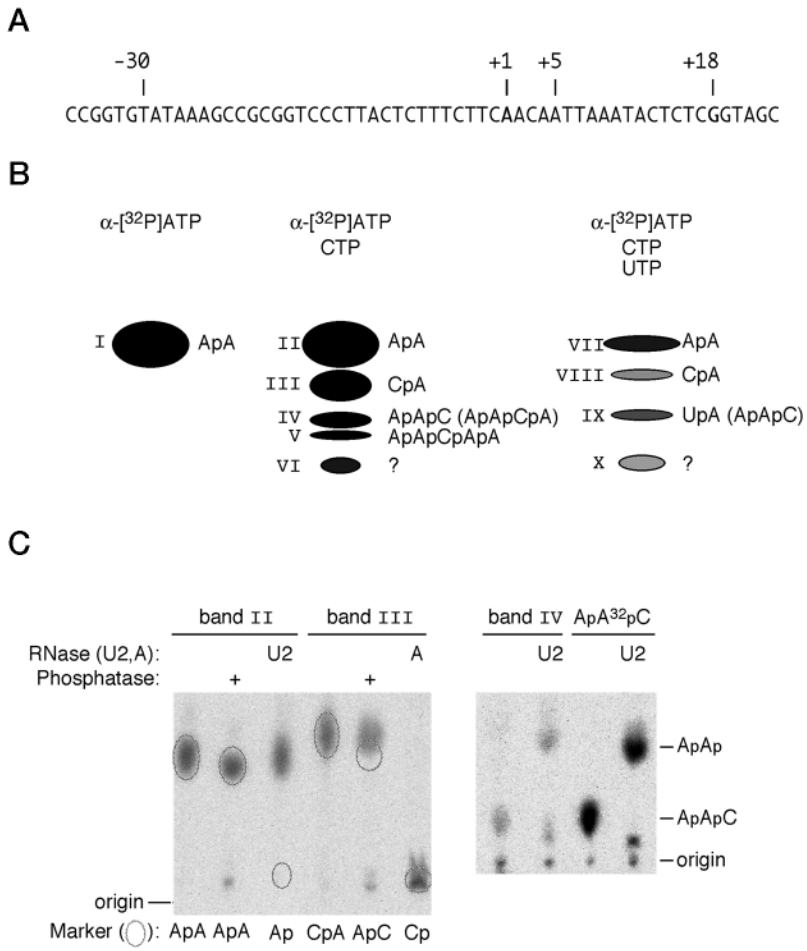

FIG. 1. **Identification of abortive transcripts of the *SUP4* tRNA gene.** *A*, initial transcribed sequence of the *SUP4* tRNA gene. The site of transcriptional initiation is designated as +1. *B*, drawing and identification of the low molecular weight reaction products in the 30-min samples of Fig. 2*A*. Oligonucleotides are identified to the *right* of each band; minor or trace components are specified in *brackets*. Bands VI and X (a *question mark* represents unidentified) were inconsistently observed and apparently arise from incomplete phosphatase cleavage. *C*, thin layer chromatographic analysis of bands II, III, and IV. Material from band II, III, or IV or *in vitro* synthesized ApApC was incubated overnight at 37 °C with 1 unit of shrimp alkaline phosphatase, 1 unit of RNase U2, or 50 μg/ml RNase A, as indicated *above* each *lane*. Reaction mixtures containing material from bands II and III were mixed with the unlabeled markers indicated *below* each *lane*, spotted onto a polyethyleneimine thin layer film, and developed with 0.16 M LiCl as solvent. Reaction mixtures with material from band IV and labeled ApApC were spotted on a separate thin layer film and separated with the same solvent.

Fig. 2*A* displays a time course of the accumulation of abortive and productive transcripts following the addition to preformed TFIIIC-TFIIIB-pol III-DNA complexes of 25 μM [α-^{32}P]ATP, 25 μM [α-^{32}P]ATP and CTP, or 25 μM [α-^{32}P]ATP, CTP, and UTP, with 100 μM 3′O-MeGTP present to prevent elongation past G18 (due to the presence of trace ITP). It can be seen readily that short oligonucletotide products accumulate with time in the presence of ATP or ATP plus CTP but that their abundance diminishes greatly when UTP is also present. The same products were also generated when a 270-bp restriction fragment containing the TA-30 variant of the *SUP4* tRNA gene replaced the entire plasmid as DNA template; synthesis of these small products was absolutely dependent on the presence of both TFIIIB and TFIIIC (data not shown); both findings indicate that these products were derived from specific initiation at the *SUP4* gene. The expected product from the reaction containing only ATP is ApA (after removal of 5′-terminal phosphates with alkaline phosphatase). We were therefore surprised that reaction mixtures containing ATP- and CTP-generated products that migrated ahead of the presumed ApA product on 20% polyacrylamide gels. The products generated after 30 min of synthesis were eluted from their gels and further analyzed as described under "Materials and Methods." The resulting identifications are summarized in Fig. 1*B* and in the *right side* of Fig. 2*A*. Only ApA was synthesized in the presence of ATP (Fig. 2*A*) and accumulated at a constant rate for 30 min (Fig. 3*A*). There was no evidence of slippage generating products containing more than two A residues (5).

In the presence of both ATP and CTP, pol III also synthesized ApA, which accumulated in a linear fashion over a 30-min period at approximately 80% of the ATP-alone rate (Figs. 2*A* and 3*A*). The next most abundant product proved to be CpA, which accumulated at one-third the rate of ApA (Figs. 2*A* and 3*A*). This was perplexing, since no full-length *SUP4* transcripts or ternary transcription complexes arrested at C17 can be ascribed to initiation occurring at either bp −1 or bp +3 (5, 43, 47, 51, 53), whereas productive initiation at bp −1 is allowed when transcription is primed with the dinucleotide CpA (Ref. 53; data for the pTA-30 template not shown). We argue below that the CpA product results from exonucleolytic retraction during abortive initiation. A barely resolved doublet of bands below CpA was identified as ApApC and ApApCpApA (Fig. 2*A*; a trace, but negligible, amount of ApApCpA was also contained in the ApApC band). ApApC and ApApCpApA accumulated linearly for 30 min at ~5 and ~1.3% of the rate of ApA, respectively (Fig. 3*A*), but their initial rates of accumulation during the first minute of synthesis were approximately 4-fold higher (Fig. 3*A*, *inset*).

The addition of UTP and 3′O-MeGTP to allow pol III to extend an RNA chain to G18 greatly reduced abortive initiation and generated the expected G18 ternary transcription complex (Fig. 2*A*). However, we also observed bands corresponding in size to transcripts with 3′-ends at U16 and U14 that had not been seen previously in reactions lacking 3′O-MeGTP (48, 51). (Weaker bands corresponding to C17, C15, and C13 could also be detected in the original autoradiogram.) The accumulation of all of the ≥14-nt RNA chains ceased after 4 min of synthesis; each displayed a half-time of formation of 1–2 min. We consider each of these transcripts to be productive, since they are not synthesized reiteratively with time. Accordingly, their yields have been summed in Fig. 3*B*. ApA and CpA continued to be synthesized reiteratively for 30 min but at one-eighth the rate

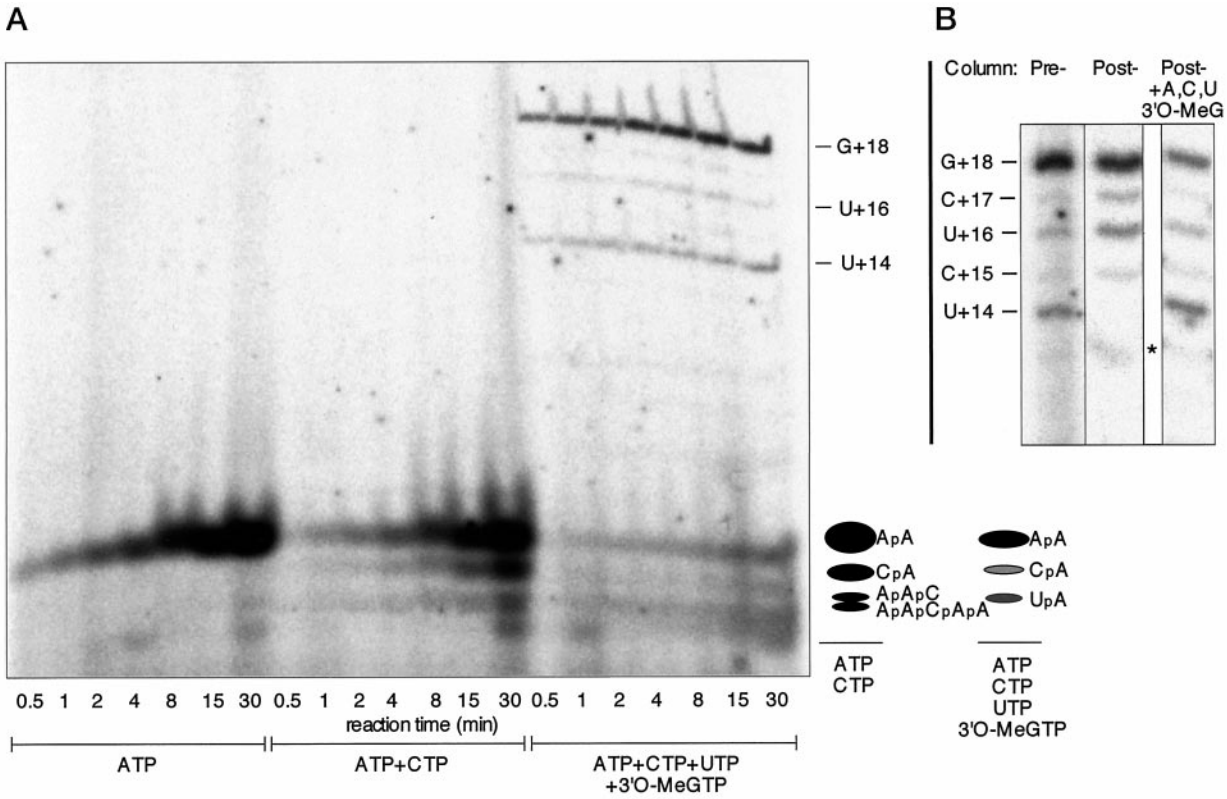

FIG. 2. **The abortive initiation phase of transcription of the *SUP4* tRNA gene.** *A*, time course of abortive and productive initiation. Transcription complexes were preformed, and transcription was started by adding ATP, ATP plus CTP, or ATP plus CTP plus UTP plus 3′O-MeGTP to begin transcription for the times specified at the *bottom* (for details, see "Materials and Methods"). Abortive and productive initiation products are identified on the *right*. *B*, nucleolytic retraction of the G18 elongation complex in the presence of ATP, CTP, UTP, and 3′O-MeGTP. Stable elongation complexes were formed for 15 min in the presence of [α-^{32}P]ATP, CTP, UTP, and 3′O-MeGTP (*Pre-*), purified by chromatography on Sepharose CL2B (*Post-*), and incubated for an additional 15 min in the presence of ATP, CTP, UTP, and 3′O-MeGTP prior to gel electrophoresis. Equal volume fractions of the sample were loaded in each lane. Productive and abortive products are identified at the *left*. (The band marked with an *asterisk* is a gel artifact and not an RNA product.)

of the initial minute (Fig. 3*B*).

Upon further analysis, the product co-migrating with ApApC in the ATP plus CTP plus UTP plus 3′O-MeGTP reaction (Fig. 2*A*) proved to be UpA; only negligible levels of ApApC were present in this band (see "Materials and Methods"). If this band is derived from the initial *SUP4* transcript, it must be generated by nucleolytic retraction, either from nt 8 to nt 6 or from nt 12 to nt 10. Production of UpA was linear for 30 min, at about 60% of the rate of ApA synthesis in the same reaction (analysis not shown). The presence and reiterative synthesis of UpA suggested that the U16 and U14 productive transcripts may be derived from nucleolytic retraction from G18 in dinucleotide increments (10, 48). Retraction past U14 to A12 and A10 followed by resynthesis back to U14 and beyond would repetitively generate labeled UpA.

To explore retraction under the conditions of the time course analysis shown in Fig. 2*A*, RNA synthesis with 25 μM [α-^{32}P]ATP, CTP, and UTP and 100 μM 3′O-MeGTP was terminated after 15 min by the addition of EDTA (which also prevents the Mg^{2+}-dependent nucleolytic retraction; Ref. 48), and part of the reaction mixture was chromatographed on Sepharose CL2B, which excludes plasmid DNA and stable ternary transcription complexes but includes unbound proteins as well as released reaction products and substrates (52). *Lane 1* of Fig. 2*B* displays the RNA products present in the reaction mixture prior to chromatography. *Lane 2* displays the plasmid-containing excluded fraction: RNA with 3′ ends at G18, C17, U16, and (partly) at C15 was retained in these presumptive ternary transcription complexes, but shorter transcripts were not stably retained. This is surprising, since a C10 ternary complex formed on the 5 S ribosomal RNA gene is stable to chromatography under comparable conditions (52), whereas the presumptive U14 *SUP4* complex is not. The difference may reflect different stabilities of the corresponding RNA-DNA hybrids. (The last 6 nucleotides of the C10 transcript of the 5 S RNA gene are G or C, whereas 5 of the last 6 nt of the U14 transcript of the *SUP4* gene are A or U.) A reaction mixture with 25 μM ATP and 25 μM CTP was subjected to the same chromatography; no transcripts were stably retained in the pol III-DNA complex (data not shown).

When unlabeled ATP, CTP, UTP, 3′O-MeGTP, and $MgCl_2$ were added to the Sepharose-purified ternary transcription complexes arrested at bp +18 (*lane 2*) so as to match the precolumn reaction conditions, much of the label (from [α-^{32}P]AMP) in the G18, C17, and U16 nascent RNA was seen to disappear (*lane 3*), converting partly to the U14 transcript and partly to shorter products, including UpA and ApA (data not shown). It is conceivable that the placement of chain elongation-arresting 3′O-MeGTP at the 3′-end of the RNA chain initiates a retraction-resynthesis mode for RNA polymerase that results in the reiterative production of UpA. Reiterative synthesis of dinucleotide retraction products has also been observed for pol III as it transcribes through sites of strong transcriptional pausing (10).

The time course analysis in Fig. 2*A* and similar experiments allowed us to estimate the average number of abortive transcripts that were made by a pol III-promoter complex in the process of forming a productive elongation complex. Fig. 3, *B* and *C*, show the accumulation of RNA chains of length ≥14 nt, representing the productive elongation complexes. The contin-

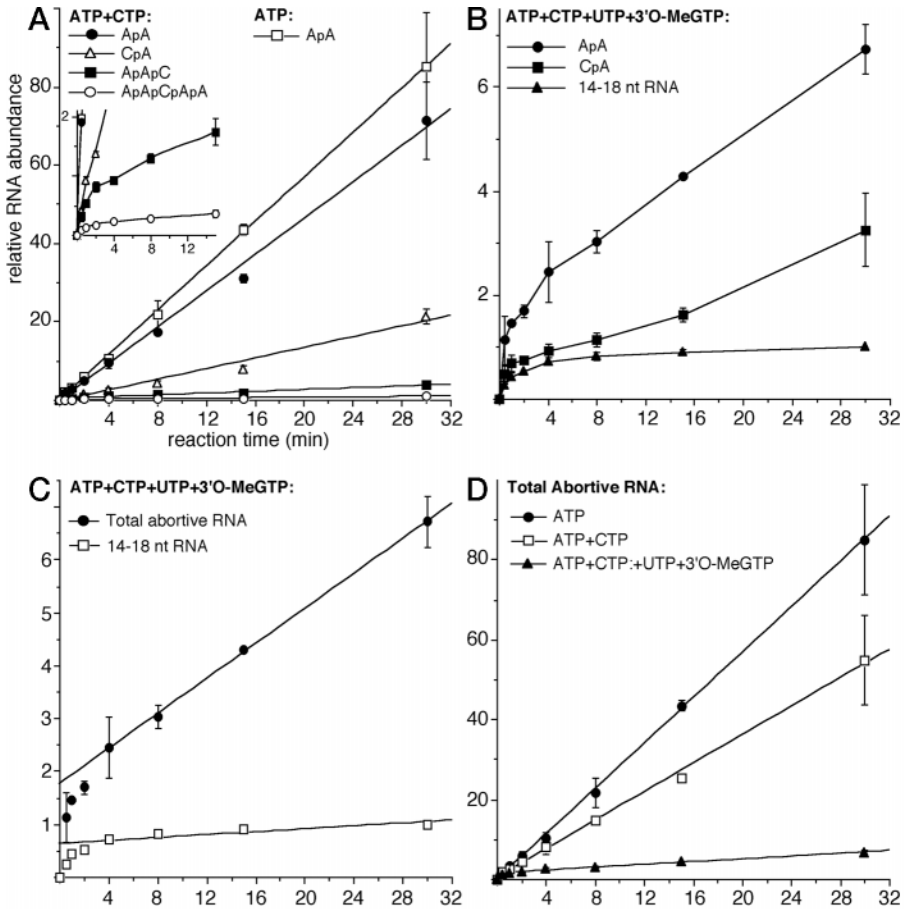

FIG. 3. **Accumulation of abortive and productive transcripts.** Bands corresponding to ApA, CpA, ApApC, ApApCpApA, UpA, and 14–18-nt RNAs were quantified by phosphor image analysis after gel background correction from three time course experiments identical with Fig. 2*A*. Data are expressed as molar yields normalized to the sum of 14–18-nt RNA synthesized after 30 min with ATP plus CTP plus UTP plus 3′O-MeGTP. The averages from three experiments are plotted with their S.E. *A*, accumulation of abortive initiation products synthesized in the presence of ATP or ATP plus CTP. ATP-only reaction product was as follows: ApA (□). ATP plus CTP reaction products were as follows: ApA (●), CpA (△), ApApC (■), and ApApCpApA (○). *B*, accumulation of abortive and productive (14–18-nt) transcripts in the presence of ATP, CTP, UTP, and 3′O-MeGTP. ●, ApA; ■, CpA; ▲, 14–18-nt RNA. *C*, abortive products synthesized per active transcription complex. The accumulation of ApA (●), representing total abortive output and 14–18-nt RNA (□) synthesized in reactions containing ATP, CTP, UTP, and 3′O-MeGTP between 4 and 30 min is extrapolated to time 0. The ratio of these ordinate intercepts approximates the number of rounds of abortive initiation per productive elongation complex. *D*, data for determining rates of abortive cycling by elongation-competent pol III. The sum of abortive transcripts per active pol III initiation complex synthesized in the presence of ATP (●), ATP plus CTP (□), and ATP plus CTP plus UTP plus 3′O-MeGTP (▲) is plotted. (For further discussion and analysis, see "Results.")

ued accumulation of ApA and CpA after formation of these productive complexes has reached its plateau indicates that a proportion of polymerase molecules fails to make the transition to productive RNA chain elongation. (This proportion proved to be low; see below.) Extrapolating the accumulation of abortive and productive transcripts between 4 and 30 min back to zero time should correct for this residuum, and the ratio of $t = 0$ intercepts should closely approximate the rounds of abortive initiation per productive initiation event (Fig. 3*C*). When all short RNA (including UpA) is considered to be a direct abortive product, the ratio of abortive to productive events is found to be 4.8. When only ApA and CpA are considered to be the direct products of abortive initiation, the ratio reduces to 3.2. On the basis of these values, one would predict that, in the presence of ATP and CTP only, production of ApApCpApA should represent 20–30% of the total abortive output. In contrast, ApApCpApA represented only 3% of the total at 30 s, dropping to a plateau of 1% by 8 min of synthesis (analysis not shown). Since there is no evidence for initiation by pol III at bp −1 or +3, a nucleolytic retraction origin for CpA is highly probable.

If retraction occurs preferentially in dinucleotide increments near the transcriptional start, as it does elsewhere (10, 48), then ApApCpA would yield labeled ApA and CpA, and ApApCpApA would generate labeled ApA and ApApC (or unlabeled ApC and A). For the analysis shown in Fig. 3*C*, both CpA and UpA are accordingly presumed to be products of nucleolytic retraction, while the accumulation of ApA is taken to monitor the sum of all abortive products (since it is produced on an equimolar basis from retraction of all products except ApApC and since the latter is not present at appreciable levels). Under these assumptions, there are at most 2.8 abortive events, on average, on the path to formation of each productive elongation complex. Abortive initiation events per productive elongation complex were also low (<4) in the presence of 100 or 400 μM ATP, CTP, UTP, and 3′O-MeGTP (data not shown).

On at least some prokaryotic promoters, a significant fraction of *E. coli* RNA polymerase molecules enters into a "moribund" state, competent for abortive but not for productive initiation of transcription *in vitro* (54). The fraction of such pol III complexes must be low; the decrease in the rate of accumulation of abortive products in the presence of UTP and 3′O-MeGTP (in addition to ATP and CTP) (Fig. 3*D*) indicates that 90% of promoter-bound polymerase that is competent for abortive initiation is also competent for formation of a productive elongation complex. (The 90% value is essentially invariant to alternative judgments of what constitutes an abortive or re-

traction product).

Our analysis also yields an estimate of the rate of production of abortive transcripts by elongation-competent pol III. In the presence of ATP alone, each round of ApA synthesis requires, on average, 23 s. The rate of abortive initiation is 27% lower with ATP plus CTP, relative to ATP only, each round of abortive initiation (ApA plus ApApC plus ApApCpApA; excluding CpA) requiring 37 s. This slower rate may reflect the additional involvement of nucleolytic retraction in generating ApA from ApApCpA and ApApCpApA. Low substrate concentrations (25 μM) contribute to this slow rate of abortive initiation, but even at 400 μM ATP and CTP, each round of abortive initiation was estimated to require 14 s (data not shown).

DISCUSSION

RNA polymerase III is brought to the start site of transcription through its interaction with the TFIIIB-DNA complex and retains that association in the open promoter complex (13). Because pol III executes a first round of transcription much more slowly than subsequent rounds, it has been proposed that it never leaves its template but recycles directly from termination to reinitiation on the same gene (6). One way of generating a closed transcription loop would be for pol III to remain associated with the TFIIIB-DNA complex as it elongates its short transcripts. The loss of DNA contact upstream of the start site at a relatively early step of productive RNA chain elongation on the *SUP4* tRNA gene (55) argues against this possibility; so does the energy cost of topologically unwinding 10 helical repeats of DNA if pol III and the DNA bound by TFIIIB are rotationally fixed during transcription of the *SUP4* gene.

If pol III does release from the TFIIIB-DNA complex, it would be of interest to know at what point this occurs and to what extent release from TFIIIB limits the overall rate of transcript generation. To this end, we have examined initial steps of RNA synthesis leading to the formation of a productive elongation complex for any effects that TFIIIB-pol III interactions might have on the transition to productive RNA chain elongation. We find that, like pol II and bacterial RNA polymerases, pol III undergoes a phase of abortive initiation prior to forming a stable, productive elongation complex. Abortive initiation is most clearly evident under conditions that limit the nascent RNA chain to 5 nt (Fig. 2*A*). When the missing nucleotides are provided, allowing formation of a productive elongation complex (containing an 18-nt nascent RNA chain), abortive initiation is greatly curtailed: each molecule of pol III undergoes approximately three cycles of abortive initiation prior to the formation of a productive elongation complex on average (Fig. 3*C*). Abortive initiation by pol II on the adenovirus E4 and major late promoters is also curtailed in presence of all four ribonucleoside triphosphates (28, 56). In contrast, a significant fraction of *E. coli* RNA polymerase molecules either fails to escape the abortive initiation phase at some promoters or generates more than 50 abortive products per productive transcript (21, 31, 54). A characteristic feature of abortive initiation by pol II at the adenovirus major late promoter is the absence of 2-mer abortive products when 3-mer abortive products can form and the absence of 2-mer to 4-mer abortive products when 5-mers can form (28). With pol III, ApA synthesis continues at a significant rate at the *SUP4* promoter in the presence of ATP and CTP, which allows formation of a 5-mer (Figs. 2*A* and 3*A*). At least one-third of the ApA accumulation is generated by nucleolytic retraction in dinucleotide steps, as evidenced by the concurrent production of CpA, and nearly all ApA accumulation may arise in this way.

In the presence of 25 μM ATP, CTP, and UTP and 100 μM 3′O-MeGTP, pol III forms a stable elongation complex arrested at G18 (with a half-time of formation of 1–2 min), with no sites of pausing or arrest discerned beyond 5 nt (Figs. 2*A* and 3*B*); smaller transcripts (13–17 nt) form but appear to be derived from nucleolytic retraction of the 18-mer elongation complex (Fig. 2*B*). If pol III releases from TFIIIB subsequent to synthesis of the 5-mer, it must do so rapidly (relative to elongation step times) (3). We note that the rate of formation of a productive elongation complex in our experiments is considerably slower than the 10–30 s cited by Dieci and Sentenac (6); this is largely due to the lower concentration of initiating nucleotides used in this study (25 *versus* 500 μM), but even at 400 μM NTP our observed rate of 18-mer elongation complex formation would only barely accommodate the ~30 s recycling rate of pol III in multiple-round transcription assays (6, 51).

In the presence of ATP and CTP, ApApCpApA accumulates as an abortive initiation product at approximately 1% of the rate of ApA (Fig. 3*A*), whereas the ratio of abortive products to productive elongation complexes in the presence of ATP, CTP, and UTP demands a 20–30-fold higher rate of synthesis. This discrepancy and the appearance of CpA as a major abortive initiation product attest to efficient nucleolytic retraction from bp +5. A similar phenomenon was observed with *E. coli* RNA polymerase under conditions allowing the formation of an 8-nt RNA chain, just 1 nt short of the point of σ release (32).

Nucleolytic retraction activities have been identified as components of transcription by bacterial, eukaryotic, and virus-encoded RNA polymerases (48, 57–61). Nucleolytic retraction appears to function primarily in extricating RNA polymerase from blocks to RNA chain elongation (reviewed in Refs. 40 and 62), but it has also been shown to facilitate promoter escape by *E. coli* RNA polymerase at some promoters *in vivo* as well as *in vitro* (63). The retraction that we have observed could reflect the hold that the TFIIIB-DNA complex exerts on pol III, with release occurring only after repeated retraction and resynthesis of a 5-mer nascent RNA chain. The initial burst of CpA synthesis in the presence of ATP, CTP, and UTP (Fig. 3*B*) indicates that this retraction occurs even when RNA chain elongation can proceed beyond the 5-mer. One can interpret the above retraction of *E. coli* RNA polymerase (32) as a similar response to σ, restraining RNA polymerase through its interaction with the −10 and −35 promoter elements, with retraction as a means for repeated attempts of escape from the promoter.

Nucleolytic retraction by pol III is notably slower than the rate of RNA chain elongation (10, 12, 48), whereas the rate of cleavage of ApApCpApA must vastly exceed its rate of accumulation, not only at low nucleotide concentrations (Fig. 3*A*) but also with 400 μM ATP plus CTP (data not shown). This relatively rapid retraction would readily explain a long-standing observation that the transcription bubble on the *SUP4* tRNA gene does not translocate along the DNA template in response to addition of CTP plus ATP to an open promoter complex (13). The absence of significant accumulation of ApApCpApA also reflects the observation that abortive initiation by pol III is slow; each round of abortive synthesis of ApA, ApApC, and ApApCpApA in the presence of 25 or 400 μM ATP plus CTP requires ~37 or ~14 s, respectively (Fig. 3*D*). This is considerably slower than the 1.5 s required for a round of abortive initiation by *E. coli* RNA polymerase at the λP_R promoter in the presence of 500 μM ATP and 50 μM UTP (64). The slow rate of formation of the productive elongation complex in presence of 25 μM NTPs (Fig. 3*B*) primarily stems from the preceding slow abortive initiation events. Even in the presence of much greater concentrations of NTPs (400 μM), the time required for each round of abortive initiation is approximately 3 times longer than the time required to extend the RNA chain from nt 17 to the terminator (3).

Acknowledgment—We thank E. Peter Geiduschek for advice, encouragement, support, and critical reading of the manuscript.

REFERENCES

1. Geiduschek, E. P., and Kassavetis, G. A. (1995) *Curr. Opin. Cell Biol.* **7,** 344–351
2. White, R. J. (1998) *RNA Polymerase III Transcription*, 2nd Ed., Springer-Verlag and Landes Bioscience, New York
3. Matsuzaki, H., Kassavetis, G. A., and Geiduschek, E. P. (1994) *J. Mol. Biol.* **235,** 1173–1192
4. Bardeleben, C., Kassavetis, G. A., and Geiduschek, E. P. (1994) *J. Mol. Biol.* **235,** 1193–1205
5. Dieci, G., Hermann-Le Denmat, S., Lukhtanov, E., Thuriaux, P., Werner, M., and Sentenac, A. (1995) *EMBO J.* **14,** 3766–3776
6. Dieci, G., and Sentenac, A. (1996) *Cell* **84,** 245–252
7. Shaaban, S. A., Krupp, B. M., and Hall, B. D. (1995) *Mol. Cell Biol.* **15,** 1467–1478
8. Shaaban, S. A., Bobkova, E. V., Chudzik, D. M., and Hall, B. D. (1996) *Mol. Cell Biol.* **16,** 6468–6476
9. Thuillier, V., Brun, I., Sentenac, A., and Werner, M. (1996) *EMBO J.* **15,** 618–629
10. Bobkova, E. V., and Hall, B. D. (1997) *J. Biol. Chem.* **272,** 22832–22839
11. Studitsky, V. M., Kassavetis, G. A., Geiduschek, E. P., and Felsenfeld, G. (1997) *Science* **278,** 1960–1963
12. Chédin, S., Riva, M., Schultz, P., Sentenac, A., and Carles, C. (1998) *Genes Dev.* **12,** 3857–3871
13. Kassavetis, G. A., Blanco, J. A., Johnson, T. E., and Geiduschek, E. P. (1992) *J. Mol. Biol.* **226,** 47–58
14. Popham, D. L., Szeto, D., Keener, J., and Kustu, S. (1989) *Science* **243,** 629–635
15. Sasse-Dwight, S., and Gralla, J. D. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 8934–8938
16. Chen, Y. F., and Helmann, J. D. (1997) *J. Mol. Biol.* **267,** 47–59
17. Severinov, K., and Darst, S. A. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 13481–13486
18. Li, X. Y., and McClure, W. R. (1998) *J. Biol. Chem.* **273,** 23549–23557
19. Kassavetis, G. A., Kumar, A., Letts, G. A., and Geiduschek, E. P. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 9196–9201
20. Kumar, A., Grove, A., Kassavetis, G. A., and Geiduschek, E. P. (1998) in *Mechanisms of Transcription*, Vol. 63, pp. 121–129, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
21. Carpousis, A. J., and Gralla, J. D. (1980) *Biochemistry* **19,** 3245–3253
22. Carpousis, A. J., and Gralla, J. D. (1985) *J. Mol. Biol.* **183,** 165–177
23. Hansen, U. M., and McClure, W. R. (1980) *J. Biol. Chem.* **255,** 9564–9570
24. Munson, L. M., and Reznikoff, W. S. (1981) *Biochemistry* **20,** 2081–2085
25. Krummel, B., and Chamberlin, M. J. (1989) *Biochemistry* **28,** 7829–7842
26. Luse, D. S., and Jacob, G. A. (1987) *J. Biol. Chem.* **262,** 14990–14997
27. Jacob, G. A., Kitzmiller, J. A., and Luse, D. S. (1994) *J. Biol. Chem.* **269,** 3655–3663
28. Holstege, F. C., Fiedler, U., and Timmers, H. T. (1997) *EMBO J.* **16,** 7468–7480
29. Kumar, K. P., Akoulitchev, S., and Reinberg, D. (1998b) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 9767–9772
30. Jin, D. J., and Turnbough, C. L., Jr. (1994) *J. Mol. Biol.* **236,** 72–80
31. Hernandez, V. J., Hsu, L. M., and Cashel, M. (1996) *J. Biol. Chem.* **271,** 18775–18779
32. Metzger, W., Schickor, P., Meier, T., Werel, W., and Heumann, H. (1993) *J. Mol. Biol.* **232,** 35–49
33. Kugel, J. F., and Goodrich, J. A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 9232–9237
34. Dvir, A., Conaway, R. C., and Conaway, J. W. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 9006–9010
35. Moreland, R. J., Hanas, J. S., Conaway, J. W., and Conaway, R. C. (1998) *J. Biol. Chem.* **273,** 26610–26617
36. Rasmussen, E. B., and Lis, J. T. (1995) *J. Mol. Biol.* **252,** 522–535
37. Li, B., Weber, J. A., Chen, Y., Greenleaf, A. L., and Gilmour, D. S. (1996) *Mol. Cell. Biol.* **16,** 5433–5443
38. Krumm, A., Hickey, L. B., and Groudine, M. (1995) *Genes Dev.* **9,** 559–572
39. Spencer, C. A., and Groudine, M. (1990) *Oncogene* **5,** 777–785
40. Uptain, S. M., Kane, C. M., and Chamberlin, M. J. (1997) *Annu. Rev. Biochem.* **66,** 117–172
41. Mason, P. B., Jr., and Lis, J. T. (1997) *J. Biol. Chem.* **272,** 33227–33233
42. Otero, G., Fellows, J., Li, Y., de Bizemont, T., Dirac, A. M. G., Gustafsson, C. M., Erdjument-Bromage, H., Tempst, P., and Svejstrup, J. Q. (1999) *Mol. Cell.* **3,** 109–118
43. Zecherle, G. N., Whelen, S., and Hall, B. D. (1996) *Mol. Cell. Biol.* **16,** 5801–5810
44. Tintut, Y., Wang, J. T., and Gralla, J. D. (1995) *J. Biol. Chem.* **270,** 24392–24398
45. Ellinger, T., Behnke, D., Bujard, H., and Gralla, J. D. (1994) *J. Mol. Biol.* **239,** 455–465
46. 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
47. Joazeiro, C. A. P., Kassavetis, G. A., and Geiduschek, E. P. (1996) *Genes Dev.* **10,** 725–739
48. Whitehall, S. K., Bardeleben, C., and Kassavetis, G. A. (1994) *J. Biol. Chem.* **269,** 2299–2306
49. Randerath, K., and Randerath, E. (1967) *Methods Enzymol.* **12,** 323–347
50. Brownlee, G. G. (1972) *Laboratory Techniques in Biochemistry and Molecular Biology*, Vol. 3, pp. 199–200, North-Holland Publishing Co., Amsterdam
51. Kassavetis, G. A., Riggs, D. L., Negri, R., Nguyen, L. H., and Geiduschek, E. P. (1989) *Mol. Cell. Biol.* **9,** 2551–2566
52. Kassavetis, G. A., Braun, B. R., Nguyen, L. H., and Geiduschek, E. P. (1990) *Cell* **60,** 235–245
53. Léveillard, T., Kassavetis, G. A., and Geiduschek, E. P. (1993) *J. Biol. Chem.* **268,** 3594–3603
54. Kubori, T., and Shimamoto, N. (1996) *J. Mol. Biol.* **256,** 449–457
55. Bartholomew, B., Durkovich, D., Kassavetis, G. A., and Geiduschek, E. P. (1993) *Mol. Cell. Biol.* **13,** 942–952
56. Jiang, Y., Yan, M., and Gralla, J. D. (1995) *J. Biol. Chem.* **270,** 27332–27338
57. Surratt, C. K., Milan, S. C., and Chamberlin, M. J. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 7983–7987
58. Reines, D. (1992) *J. Biol. Chem.* **267,** 3795–3800
59. Hagler, J., and Shuman, S. (1993) *J. Biol. Chem.* **268,** 2166–2173
60. Tschochner, H. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 12914–12919
61. Sastry, S. S., and Ross, B. M. (1997) *J. Biol. Chem.* **272,** 8644–8652
62. Kassavetis, G. A., and Geiduschek, E. P. (1993) *Science* **259,** 944–945
63. Hsu, L. M., Vo, N. V., and Chamberlin, M. J. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 11588–11592
64. McClure, W. R., Cech, C. L., and Johnston, D. E. (1978) *J. Biol. Chem.* **253,** 8941–8948