A Block of Transcription Elongation by RNA Polymerase II at Synthetic Sites in Vitro*

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We have previously suggested that transcription elongation by RNA polymerase II can be blocked when the nascent RNA is folded into a stem-and-loop structure followed by polyuridines. As an approach to test this suggestion in vitro, several GC-rich deoxyoligonucleotides with dyad symmetries were chemically synthesized and inserted following the adenovirus 2 major late promoter. These constructs were transcribed in vitro using HeLa whole cell extract. The transcripts of the synthetic inserts can potentially form stem-and-loop structures with destabilization energy from 0 to −48 kcal followed by 3, 5, and 8 U residues.

The results obtained show that transcription elongation is blocked by these synthetic inserts and that the extent of the elongation block is directly correlated to the stabilities of the potential stem-and-loop structure and the proceeding number of U residues. Three levels of elongation blocks were observed: a brief pause of the polymerase occurs when the RNA could be folded into a secondary structure or when there were 5–6 T residues on the sense DNA strand. An extended pause occurred when the number of T residues on the sense DNA strand was increased to 8. Transcription termination, with a partial release of the attenuated transcript occurred when a stable RNA secondary structure was formed in the sense DNA strand, following the Ad2 major late promoter (MLP). These constructs were transcribed in the HeLa whole cell extract system (WCE) (25). The results obtained show that transcription elongation is blocked by these synthetic inserts and that the extent of the elongation block is directly correlated to the stability of the stem-and-loop structure and the number of U residues proceeding them.

Transcription termination plays an important role in regulating gene expression in bacteria (1–3). The elements that are required to form a bacterial terminator are well defined. They fall into two major classes: factor-dependent and factor-independent terminators. Factor-dependent termination usually occurs at multiple sites and no common sequence has been identified. Factor-dependent termination is characterized by a GC-rich stretch of DNA with dyad symmetry followed by T residues in the sense DNA strand (for a review see Ref. 3). A wide variety of experimental results support the hypothesis that the GC-rich sequence in the DNA that enables the RNA transcript to fold into a stem-and-loop structure impedes the progress of the prokaryotic RNA polymerase, and then the instability of the dA:dU base pairing between the DNA template and the RNA transcript facilitates the release of the transcript from the template (4, 5).

As in prokaryotes, RNA polymerase II in eukaryotes is capable of prematurely terminating transcription within a gene. This mechanism has been termed a block of transcription elongation or attenuation and was shown to occur within the leader region of the yeast LEU 2 gene (6), in the long terminal repeat of HIV-1 (7), in the c-myc gene (8–10), in the c-fos gene (11), and in the c-myc gene (12). In addition, premature transcription termination at precise sites was shown to occur both in vitro in SV40 (13,14) and in vitro in the parvovirus minute virus of mice (MVM) (15, 20, 21) and in the adenovirus 2 (Ad2) (22–24). We have suggested that the termination signal, at least in the viral systems, includes in addition to a run of U residues at the 3′ of the terminated RNA, an upstream stem-and-loop structure. This would suggest that the prokaryotic polymerase and the eukaryotic polymerase II can respond to a similar transcription termination signal (16, 19).

To test this suggestion directly we have inserted several GC-rich synthetic deoxyoligonucleotides that have dyad symmetries and are proceeded by various numbers of T residues in the sense DNA strand, following the Ad2 major late promoter (MLP). These constructs were transcribed in the HeLa whole cell extract system (WCE) (25). The results obtained show that transcription elongation is blocked by these synthetic inserts and that the extent of the elongation block is directly correlated to the stability of the stem-and-loop structure and the number of U residues proceeding them.

**MATERIALS AND METHODS**

**Chemicals**—Nucleoside triphosphates, Sarkosyl, and creatine phosphate were purchased from Sigma, heparin sodium from BDH Chemicals, and [α-32P]UTP and [γ-32P]CTP (400 Ci/mmol) from Amer sham International.

**DNA Template Preparation**—Three pairs of complementary oligomers of 21, 23, and 26 nucleotides (a total of six oligomers) (see Fig. 1) were synthesized on DNA synthesizer model 380B using the phosphoramidite method (Applied Biosystems, Inc.). The full length products were purified from a 20% polyacrylamide nondenaturing gel. Complementary oligonucleotides were annealed at 80 °C followed by slow cooling to room temperature. Unphosphorylated double-stranded deoxyoligonucleotides were blunt-end-ligated into the StuI site of pSV2CAT plasmid DNA. To delete a part of the inverted repeat sequences, plasmid DNAs were digested with Smal, which cut twice at the inserted oligomers. The digested DNAs were then gel-purified and religated. Positive clones which contained a 10-bp deletion of the expected fragment were selected by sequencing. To further stabilize the original potential secondary structure, a 12-bp EcoRI linker, 5′-GGCCCTTAAAGCC-3′, (New England, BioLabs) was inserted at the unique EcoRV site of the inserted deoxyoligonucleotides.

**Note**—The abbreviations used are: MVM, parvovirus minute virus of mice; Ad2, adenovirus 2; MLP, major late promoter; WCE, whole cell extract; bp, base pair(s); PIPES, 1,4-piperazinediethanesulfonic acid; NTP, nucleoside triphosphate.

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Each clone of the original constructs was cut with either NcoI-HindIII or BglII-HindIII to produce two fragments that were inserted into the polylinker of the pGEM-MLP downstream from the Ad2-MLP. The target vector pGEM-MLP is a pGEM-1, containing a 450-bp SacI-Sacl (5634–6083) fragment of Ad2 inserted into the unique SacI-Smal sites of pGEM-1. For this, the BglII was first blunt ended by T4 polymerase, and BglII-HindIII was inserted at the HindII-HindIII-digested plasmid DNA. To insert the NcoI-HindIII fragment, an XbaI-NcoI adaptor was first inserted into the XbaI site of the polylinker of pGEM-MLP, thus creating a unique NcoI site.

In order to map the attenuated transcripts, a 14-bp XbaI-ClaI adaptor, 5'-CTAGGCTATCATGTC-3', that was synthesized and purified as described above, was inserted at the unique XbaI site of the polylinker of all pGEM-MLP-TER constructs.

Plasmid DNA was prepared by the alkali lysis method (26), and purified by Cetaphor density gradients.

**In Vitro Transcription Using HeLa Whole Cell Extract—WCE** was prepared according to Manley (27). A 20-μl reaction contained 10 μl of WCE, ~1 μg of DNA, 4 mM creatine phosphate, 500 μM ATP, GTP, UTP, 50 μM CTP, and 20 μCi of [α-32P]GTP, and transcription was performed at 30 °C. All reactions were preincubated 20 min before the addition of the nucleotide mixture. Sarkosyl or heparin was added 45 s after the addition of nucleotides. In the experiments where UTP was used as the labeled nucleotide, the reaction mixture contained 500 μM ATP, GTP, CTP, 50 μM UTP, and 20 μCi of [α-32P]UTP.

Labeling under pulse-chase conditions was done as follows: the pulse labeling was for 1 min and the mixture contained 500 μM ATP, GTP, UTP, and 20 μCi of [α-32P]CTP. For the chase, Sarkosyl 0.1–0.2% or heparin, 1 mg/ml, was added following 45 s later, by 500 μM of unlabeled CTP. The reactions were allowed to proceed for the times indicated, stopped by the addition of proteinase K buffer (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.2% sodium dodecyl sulfate, 10 mM NaCl), 200 μg/ml proteinase K, and 30 μg of tRNA, and incubated for an additional 15 min. RNA was phenol/chloroform-extracted and collected by ethanol precipitation and centrifugation.

**RNase Mapping**—[32P]RNA probes were synthesized according to Melton et al. (28). The DNA template was removed by treating the mixture with 10 units of RNase free DNase I ( Worthington) for 15 min. The mixture was extracted twice with phenol/chloroform, and the RNA was purified by a quick spin through a Sephadex G-25 column followed by ethanol precipitation and centrifugation. The RNA probe (50,000 cpm) was hybridized with 30 μg of unlabeled RNA in 30 μl containing 0.4 M NaCl, 40 mM PIPES, pH 6.7, 1 mM EDTA, and 80% formamide. The RNA was heat-denatured at 85 °C for 5 min and then transferred to 45 °C and incubated for an additional 3 h. The mixture was then diluted 10-fold with 0.3 M NaCl, 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 40 μg/ml RNase A, 20 units/ml T1 RNase and incubated for 1 h at 30 °C. The reaction was stopped by the addition of 0.4% sodium dodecyl sulfate and 50 μg/ml proteinase K and incubated for an additional 15 min. Nucleic acids were phenol/chloroform-extracted followed by a quick spin through a Sephadex G-25 column. Nucleic acids were collected by ethanol precipitation and centrifugation.

**Polyacrylamide Gel Electrophoresis**—RNA was resuspended in 10 μl of 90% formamide and analyzed on 6% polyacrylamide gels (10/40 acrylamide, 1:19) containing 7 M urea (Schwarz/Mann) and 1 × TBE (TBE: 89 mM Tris borate, pH 8.3, 89 mM boric acid, 2 mM EDTA). Electrophoresis was carried out at a constant current of 20 mA.

**RESULTS**

Construction of Plasmids for Assaying in Vitro the Involvement of RNA Secondary Structure and a Stretch of U Residues in the Block of Transcription Elongation—A variety of plasmids were constructed for assessing the ability of different synthetic deoxyoligonucleotides with dyad symmetries followed by various lengths of T residues in the sense DNA strand to block RNA polymerase II transcription elongation in vitro. For the details of plasmids construction see under "Materials and Methods." Only a small portion of the constructs that have been tested for their ability to block transcription elongation can potentially form RNA secondary structures with the following destabilization energy and number of consecutive U residues immediately following the RNA secondary structures: −48 kcal/mol U residues, −48 kcal/mol U residues, −48 kcal/mol U residues, −48 kcal/mol U residues, −23 kcal/mol U residues, −23 kcal/mol U residues, 0 kcal/mol U residues. Note that because the inserted deoxyoligonucleotides are of variable lengths the distance between the transcription start site and the elongation block site, if it occurs immediately following the RNA secondary structures, can vary within a few nucleotides. Fig. 2 shows schematics of two constructs that were tested in the present study. The two constructs are almost identical and they differ only by the length of the SV46 DNA fragment into which the synthetic deoxyoligonucleotides were inserted: BglII-HindIII in Fig. 2A as compared to NeoI-HindIII in Fig. 2B (see under "Materials and Methods" for details). The plasmids were linearized by Nael or NheI. The lengths of the expected runoff and attenuated transcripts are also indicated in Fig. 2. Note that the distance between the transcription start site and the potential elongation block site is different between the two plasmids. Since preliminary results showed no distance effect on the elongation block (results not shown) the constructs were designated only by the stability of the potential RNA second-
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The Potential RNA Secondary Structure and the Run of U Residues Contribute to the Efficiency of the Elongation Block—
In the following experiments moderate concentrations of Sarkosyl or heparin were included in the WCE system. The inclusion of these chemicals in the WCE system inhibits transcription initiation but not elongation by eukaryotic RNA polymerase I. Moreover, in the WCE transcription system Sarkosyl and heparin cause pausing of polymerase II. In the following experiments, moderate concentrations of Sarkosyl or heparin were included in the WCE system. The expected positions (in nucleotides (nt)) of the runoff and attenuated RNA (Alt. RNA) are indicated.

The synergistic nature of the secondary structure and the stretch of U residues can further be seen in Fig. 3, D and E, that show a comparison of transcription kinetics of two constructs: –23 kcal/8 U residues and –48 kcal/8 U residues. With both constructs an attenuated RNA is recognizable at all incubation times, including following a chase for 30 min with a high CTP concentration. However, the efficiencies of the elongation blocks, as reflected by the intensity of the attenuated RNAs (filled arrowheads), as compared to the runoffs (open arrowheads) clearly shows that it is higher with the –48 kcal/8 U residues construct as compared with the –23 kcal/8 U residues construct. Scanning the autoradiogram for determining the ratio of the attenuated to the runoff RNA (Fig. 3, D and E, lanes 1+30) revealed that the –48 kcal/8 U residues signal blocked elongation three times more effectively than the –23 kcal/8 U residues signal.

It is also evident that the increase in the number of U residues from 5 to 8 (compare Fig. 3, B and D) without changing the potential stem-and-loop structure (–23 kcal) had a pronounced effect on the efficiency of the elongation block. Thus, whereas, with the –23 kcal/5 U residues construct the attenuated RNA is chased into runoff transcript (Fig. 3B) with the –23 kcal/8 U residues construct. Scanning the autoradiogram showed that the shift of the attenuated RNA is chased into runoff transcript (Fig. 3D). We therefore conclude that both the potential stem-and-loop structure and the run of U residues contribute to the efficiency of the elongation block.

Mapping of the Attenuated RNA Transcripts and Confirmation of the Existence of RNA Secondary Structure—In order to map the attenuated RNA a fragment of 14 base pairs was inserted in all templates between the transcription start site and the site of the elongation block (see under “Materials and Methods”). If the attenuated RNA originates from the Ad2-MLP, in the extended construct both the runoff and the attenuated transcripts bands should shift upward by the corresponding 14 nucleotides. Fig. 4C shows that the expected shift of the attenuated and runoff bands is clearly seen. The attenuated RNA therefore originates from the Ad2 MLP and terminates immediately following the RNA secondary structure. The expected 14-nucleotide shift was observed with the other constructs used throughout this study.

We also attempted to map the in vitro synthesized RNA directly using RNase mapping (28). For the probe synthesized by SP6 polymerase (see Fig. 1). The runoff transcript and the attenuated RNA should protect 150 and 120 nucleotides of the labeled RNA probe, respectively. The expected positions of the protected labeled RNA probe by the runoff and attenuated RNA are designated by open and filled arrowheads, in Fig. 4A. The expected lengths of protection were not seen, instead other bands of 103–107 and 90 nucleotides were found.

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We suspect that this is due to the fact that both the probe and the RNA to be mapped are in a strong secondary structure conformation and thus only partially hybridized. The 103-107- and 90-nucleotide bands could result if the labeled RNA probe hybridizes to the RNA to be mapped as is shown in Fig. 4B and the RNase nicks this structure within the loop and at the base of the stem. In a control experiment, runoff transcripts with no potential secondary structure fully protected the labeled riboprobe and thus allowed mapping of the runoff RNA to be mapped are in a strong secondary structure at the base of the stem. In a control experiment, runoff transcripts with no potential secondary structure fully protected the labeled riboprobe and thus allowed mapping of the runoff RNA to be mapped are in a strong secondary structure at the base of the stem. In a control experiment, runoff transcripts with no potential secondary structure fully protected the labeled riboprobe and thus allowed mapping of the runoff RNA to be mapped are in a strong secondary structure at the base of the stem. In a control experiment, runoff transcripts with no potential secondary structure fully protected the labeled riboprobe and thus allowed mapping of the runoff RNA to be mapped are in a strong secondary structure at the base of the stem. In a control experiment, runoff transcripts with no potential secondary structure fully protected the labeled riboprobe and thus allowed mapping of the runoff RNA to be mapped are in a strong secondary structure at the base of the stem. In a control experiment, runoff transcripts with no potential secondary structure fully protected the labeled riboprobe and thus allowed mapping of the runoff RNA to be mapped are in a strong secondary structure at the base of the stem. In a control experiment, runoff transcripts with no potential secondary structure fully protected the labeled riboprobe and thus allowed mapping of the runoff RNA to be mapped are in a strong secondary structure at the base of the stem. In a control experiment, runoff transcripts with no potential secondary structure fully protected the labeled riboprobe and thus allowed mapping of the runoff RNA to be mapped are in a strong secondary structure at the base of the stem. In a control experiment, runoff transcripts with no potential secondary structure fully protected the labeled riboprobe and thus allowed mapping of the runoff RNA to be mapped are in a strong secondary structure at the base of the stem. In a control experiment, runoff transcripts with no potential secondary structure fully protected the labeled riboprobe and thus allowed mapping of the runoff RNA to be mapped are in a strong secondary structure at the base of the stem. In a control experiment, runoff transcripts with no potential secondary structure fully protected the labeled riboprobe and thus allowed mapping of the runoff RNA to be mapped are in a strong secondary structure at the base of the stem.

The Efficiency of the Elongation Block Can Be Modulated by the Number of T Residues at the Block Site—An examination of the sequence on the sense strand in the vicinity of the inserted synthetic deoxylignonucleotide revealed additional T-rich sequences (see Fig. 5C where the T sequences are represented as U residues in the RNA transcript). In addition to the 3 T, 5 T, and 8 T residues that are part of the inserted sequences, there are stretches of 6 T residues upstream and 4 T residues downstream of the inserted synthetic sequences (in the SV40 sequences). Grass et al. (31) using a similar system found several termination sites in the SV40 sequences including the sites of the 6 and 4 U residues (see Fig. 5C). In order to examine directly the effect of stretch of U residues on termination, we transcribed a construct which had no secondary structure inserted, only an insert containing 8 U residues. In Fig. 5A, the 148- and 183-nucleotide bands represent the location of an RNA transcript which reached the 6 or 4 consecutive uridines (from the SV40 sequence) in the transcript (filled arrowheads), while the 168-nucleotide band represents a transcript which reached the 8 U residues of the insert. It is apparent that in the present in vitro system, a stretch of 6 consecutive uridines led to a brief pause of the polymerase which was chased after 5 min, whereas a stretch of 4 consecutive uridine residues did not cause a noticeable pausing of the polymerase. However, 8 consecutive uridine residues had a more pronounced effect. Notice that although the intensity of the 168-nucleotide band decreases gradually with the incubation time, it is still recognizable even after 50 min of incubation.

We were able to enhance the accumulation of the RNA at the 8 U residues by adding RNA secondary structure of -48 kcal (Fig. 5B). The 188-nucleotide transcript, which represents an RNA with a 3' terminus within the 8 U residues, is present at all the time points examined. Again, a brief pause at the 6 U residues led to an RNA band of 148 nucleotides. A comparison of Fig. 5, A and B, shows that, whereas with the 0 kcal/8 U residues, at the 50-min time point <10% of the radioactivity is present at the attenuated RNA, with the -48 kcal/8 U residues construct >25% of the radioactivity is present in the attenuated RNA, indicating that there is a synergistic effect between the stem-and-loop structure and U residues on the elongation block.

The Elongation Block Is Temperature-dependent—Based on the above studies we suggest that premature termination of transcription at the inserted sequence is a two-step event involving first pausing of the polymerase at the block site, followed by the release of the transcript due to the instability of the dArU interaction (5). If instability of the dArU interaction is a dominant part of termination at this site it is expected that as the temperature of the reaction is increased, more attenuated RNA will accumulate. We compared the amount of attenuated RNA transcripts obtained when transcription elongation was carried out at 30, 37, 40, and 45 °C for 50 min (Fig. 6). The temperature of the reactions was only increased after adding the "chase" solution in order to insure that the extent of preinitiation complex formation and the degree of pulse labeling was the same for each reaction. It is apparent that the transcription temperature has a strong effect on the level of the elongation block. With increased temperature there is a gradual increase in the amount of attenuated RNA. A comparison of the amount of attenuated and runoff transcripts at 30 and 45 °C shows that the attenuated transcripts account for 25% of the total transcription at 30 °C as compared to 60% at 45 °C. Interestingly, a similar effect of temperature on the elongation block was observed in

![Fig. 3. Comparison of transcription kinetics of constructs that differ in the stabilities of the transcripts potential secondary structures and in the number of consecutive uridine residues. In A the construct was linearized with NcoI (see Fig. 2A). In B-E the constructs were linearized with NcoI (see Fig. 2B). Preincubation of the indicated linearized constructs, transcription initiation, addition of 0.1% Sarkosyl, and transcription elongation for times as indicated or labeling under pulse-chase conditions (1-min pulse, 30-min chase) were as described under "Materials and Methods." The reactions were stopped at the indicated times and the RNA was extracted and analyzed by polyacrylamide gel electrophoresis as described under "Materials and Methods."
FIG. 4. Attempts to map the attenuated RNA. A, the -48 kcal/8 U residues (-48 kcal/8 U) construct was linearized with NheI (see Fig. 2B). Preincubation with WCE, transcription initiation with four unlabeled rNTP (500 μM), addition of Sarkosyl, and further incubation for 40 min were done as described under "Materials and Methods." RNA was extracted with phenol/chloroform, treated with DNase (RNase-free), phenol-extracted, and ethanol-precipitated. Labeled riboprobe was synthesized using SP6 polymerase. The labeled riboprobe is complementary to the HindIII-NcoI fragment (see Figs. 1 and 4B). The riboprobe protection assay was done as described under "Materials and Methods." Protection of the full length NcoI-HindIII fragment should yield an RNA band of 150 nucleotides (nt) (see B), and the expected position of this band on the gel is indicated by an open arrowhead in A. RNA that terminate at the U-rich stretch should protect 120 nucleotides of the riboprobe (see B), and the expected position of this RNA band on the gel is indicated by a filled arrowhead in A. The arrows indicate the sizes of the labeled riboprobe obtained if the ribonuclease cuts at the loop and base of the stem as indicated in B. B, schematic presentation of a hybrid between the labeled riboprobe and unlabeled RNA synthesized in the WCE. The labeled and unlabeled RNA transcripts are shown in their hairpin structures with the stretches of rU-rA. The lengths, in nucleotides, of the protected entire riboprobe (150 nucleotides) and the portion protected by the attenuated RNA (120 nucleotides) are indicated. The arrows and numbers indicate the interpretation of the positions of the cuts and length, in nucleotides, of the RNA produced that correspond to the bands indicated by arrows in A. C shows the mapping of the attenuated RNA and runoff transcripts by inserting a 14-bp adaptor in the XbaI site of the pGEM-1 polylinker. The polylinker is located between the Ad2 MLP and the inserted SV40 sequences (see Fig. 1). Lane R shows the lengths, in nucleotides, of the transcripts synthesized on the regular -48 kcal/8 U residues construct cut with NaeI (see Fig. 2M), and lane E shows the lengths of the transcripts produced on the extended construct that contains the 14-bp adaptor.

A Substantial Fraction of the Nascent RNA Is Released from the -48 kcal/8 U Residues Template at the Elongation Block Site—Two approaches were used in order to verify whether the attenuated RNA transcript synthesized on the -48 kcal/8 U residues template results from pausing of the polymerase or from transcription termination. We first examined whether the elongation block is reversible when the Sarkosyl concentration is diluted 10-fold. This approach was used by Hawley and Roeder (29) and by Kessler et al. (32) in their studies on the elongation block in the Ad2-MLP system. In the Ad2-MLP system, dilution of the Sarkosyl concentration is diluted 10-fold. This approach was used by Hawley and Roeder (29) and by Kessler et al. (32) in their studies on the elongation block in the Ad2-MLP system. In the Ad2-MLP system, dilution of the Sarkosyl concentration caused an almost complete chase of the attenuated RNA into the runoff transcript (29, 32). Fig. 7A (middle lane) shows that dilution of the Sarkosyl concentration from 0.2 to 0.02%, resulted in only a partial reversal of the elongation block at the stretch of 8 U residues. This can be seen by comparing lanes 0.2-0.02 and 0.2-0.2. This experiment does not exclude the possibility that the 0.2% Sarkosyl caused an irreversible pausing of the polymerase rather than transcription termination which is characterized by the release of the nascent RNA from the template.

In the second approach transcription elongation in the presence of 0.1% Sarkosyl was performed on a circular -48 kcal/8 U residues template. After 40 min of incubation transcription was stopped by the addition of 10 mM EDTA to the reaction mixture which was then layered onto a 5-30% (w/v) sucrose gradient. After centrifugation, 32 fractions were collected and each fraction was analyzed by gel electrophoresis under denaturing conditions. The attenuated RNA of about 185 nucleotides was found in both the top fractions of the gradient as a free RNA and in the middle of the gradient together with long RNA transcripts where the circular DNA sedimented (Fig. 7B). The transcripts which sedimented with the circular DNA were probably nascent RNA attached to the
transcription complex (TC). In control experiments with constructs whose attenuated RNA is chased into runoff transcripts no free RNA was found at the top of the gradient. We conclude that about 70% of the attenuated RNA from the −48 kcal/8 U residues plasmid is released from the template and represent the level of termination in this system. The remaining 30% is attached to the template and represents pausing of the polymerase.

**Dilution of the Reaction during the Elongation Step Enhances the Elongation Block to a Similar Extent as the Presence of Sarkosyl**—One explanation for the enhancement of the elongation block by Sarkosyl and heparin is that both chemicals inactivate a readthrough or a stimulating factor. If this putative stimulating factor binds to the RNA polymerase during transcription elongation then dilution of the transcription reaction during the elongation step may reduce the binding of the factor to the polymerase and thus enhance the elongation block at the attenuation site. To investigate this possibility the 0 kcal/8 U residues construct was incubated in vitro for 1 min in the presence of [α-32P]CTP, Sarkosyl to 0.2% was added during the chase with 500 μM unlabeled CTP. Indeed, the dilution of the reaction mixture during the elongation step enhanced the synthesis of the attenuated RNA by approximately the same level as did the addition of 0.2% Sarkosyl (compare lanes b in Fig. 8, A and B). In both cases, the attenuated RNA represents about 50% of the total RNA synthesized. A similar enhancement was observed when the same protocol was used with the other constructs. The attenuated RNA in the dilution protocol remained the same length, while that of the Sarkosyl protocol is about 5 nucleotides longer. At present we do not have an explanation for this phenomenon.

**Fig. 5.** Comparison of transcription kinetics of constructs containing 8 U residues with and without potential RNA secondary structure preceding them. Preincubation of the constructs 0 kcal/8 U residues (0 kcal/8 U) in A and −48 kcal/8 U residues (−48 kcal/8 U) in B linearized with NaeI (see Fig. 2B) with WCE, transcription initiation, addition of heparin (1 mg/ml), and transcription elongation were as described under “Materials and Methods.” At the indicated times RNA was extracted and analyzed by polyacrylamide gel electrophoresis. C is a schematic showing in the hatched box the inserted synthetic oligonucleotides and in the open boxes the number of consecutive uridine residues in the vicinity of the inserted synthetic oligonucleotides. The numbers above the boxes show the calculated lengths, in nucleotides (nt), of potential transcripts that stop at the first and last U residues. Where two numbers appear, those in parentheses pertain to the transcripts in A and the others to the transcripts in B. The locations, in the gels, of the potential transcripts that stop at the first U residue of the boxes (shown in C) are indicated by filled arrowheads and the location of the runoff by open arrowheads.

**Fig. 6.** Increasing transcription temperature enhances the elongation block at the inserted sequences. Preincubation of the −48 kcal/8 U residues plasmid linearized with NaeI (see Fig. 2B), transcription initiation and addition of heparin (1 mg/ml) was as described in “Materials and Methods.” Labeling with [α-32P]CTP was done for 1 min at 30 °C. The reaction mixtures were transferred to the indicated temperatures and transcription continued, in the presence of 500 μM of the four rNTP, for 40 min. RNA was extracted and analyzed by polyacrylamide gel electrophoresis. Att. RNA, attenuated RNA.
the number of U residues proceeding them.

The types of elongation blocks that we observed can be divided into three levels: a brief pause of the transcription complex, an extended pause of the transcription complex, and transcription termination. The brief pause of the transcription complex occurs at sites where the RNA can potentially fold into a stem-and-loop structure. The brief pause is quickly relieved and full length transcripts are synthesized. It may be caused by a physical constraint imposed on the transcription complex transversing the stem-and-loop structure of the RNA (3). However, the involvement of DNA secondary structure is also possible. The extended pause occurs at a T-rich region in the sense DNA strand. The duration of the extended pause is directly dependent on the number of T residues. In the present in vitro system 6 consecutive T residues caused the transcription complex to pause for about 5 min while 8 consecutive T residues dramatically enhanced the pause of the transcription complex to more than 50 min.

A similar observation was made when the prokaryotic E. coli RNA polymerase was used to transcribe synthetic termination signals in vitro. Although termination occurred at the same site on two templates containing 10 or 20 consecutive T residues, the efficiency of termination was significantly enhanced with the extended T-stretch (33). It appears that the
polymerase can respond to sequences downstream of the actual termination site. Similarly, termination within the trp operon is significantly reduced when the number of T residues at the attenuation site are reduced (34, 35).

That consecutive T residues in the sense DNA strand can lead to transcription termination has been observed in several genes that are transcribed by RNA polymerase II. In the gastrin gene and in yeast genes a T-rich region in the sense DNA strand is at least a part of the termination signal (36–38). However, when the T-rich sequence is interrupted by dC or dG following 5 T residues as in the sequence TTTTTGTTTGGTTT, neither pausing nor termination of transcription within this site in vitro was observed (32).

The third elongation block observed in the present study can be defined as true transcription termination because a major fraction of the nascent RNA is released from the template. It was found to occur at a U-rich stretch in the RNA when it follows a stem-and-loop structure of 84 kcal. Although at first sight the effect of the stem-and-loop structure on the elongation block appears to be minimal (see for example Fig. 3A) it has a pronounced effect when it is followed by a stretch of U residues in the RNA transcript. Thus, for example, 5 U residues following a stem-and-loop structure of 23 kcal lead to a reversible block of elongation while the same number of U residues following a stem-and-loop structure of 48 kcal lead to an irreversible block of elongation. It seems, therefore, that the stem-and-loop structure and the stretch of U residues are two elements of a termination signal recognized by the eukaryotic RNA polymerase II. There is at least one mechanism for transcription termination shared by prokaryotes and eukaryotes. In the present study, the elongation reaction prevent the association of a putative readthrough factor with RNA polymerase. In support of this explanation the observation that deletion of the stimulatory factor TFIIIS from a fractionated HeLa nuclear extract results in a continued pausing of the polymerase 185 nucleotides downstream from the Ad2-MLP. The replacement of the stimulatory factor TFIIIS to the extract relieved the pause (42, 43). Binding of the readthrough factor to the polymerase thus enables the polymerase to transverse through possible block sites. In the present in vitro system, the stimulatory factor TFIIIS has no effect on the elongation block. However, we have isolated and partially purified a stimulating activity from HeLa and monkey cells that when added following transcription initiation prevented the elongation block and enhanced transcription elongation. That Sarkosyl or heparin enhance the elongation blocks are true termination sites is supported by the observations that with Ad2 and MVM systems the attenuated RNAs were identified and their 3' ends were mapped in vivo to the same sites where Sarkosyl or heparin enhance the elongation blocks in vitro (21, 24).

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