JOURNAL OF VIROLOGY, Sept. 1991, p. 4910–4918
0022-538X/91/094910-09$02.00/0

Vol. 65, No. 9

Transcriptional Elongation by Purified RNA Polymerase II Is Blocked at the *trans*-Activation-Responsive Region of Human Immunodeficiency Virus Type 1 In Vitro

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Received 14 December 1990/Accepted 4 June 1991

It has previously been shown that the human immunodeficiency virus type 1 (HIV-1) *trans*-activation-responsive region (TAR) is contained in a stem-loop RNA structure. Moreover, the interaction of the RNA secondary structure with Tat, the *trans*-activator protein, seems to play a role in activation of transcription initiation and in preventing transcription attenuation. In this work, we have studied the ability of the HIV-1 TAR stem-loop to act as a specific attenuation signal for highly purified RNA polymerase II. We developed an in vitro system using dC-tailed DNA fragments of HIV-1 to study transcriptional control in the HIV-1 LTR. We have found that transcription in this system yields an attenuator RNA whose 3′ end maps to the end of the TAR stem-loop, approximately 60 to 65 nucleotides downstream of the in vivo initiation site. Furthermore, transcription attenuation occurs only under conditions which cause displacement of the nascent transcript from the template DNA strand, thus allowing the RNA to fold into secondary structure. Evidence is provided that the purified polymerase II indeed recognizes stable RNA secondary structure as an intrinsic attenuation signal. The existence of this signal in the TAR stem-loop suggests that in vivo an antiattenuation factor, probably Tat, alone or in combination with other factors, acts to relieve the elongation block at the HIV-1 attenuation site.

Recently, it has been shown that eukaryotic RNA polymerase II, like the prokaryotic polymerase, can pause or terminate both in vivo and in vitro transcription within viral (1, 3, 19, 23, 26, 31, 37, 38, 40, 45) and cellular (4, 8, 9, 16, 24) genes and thus modulate downstream gene expression. This mechanism is termed attenuation (47). To date, however, little is known about the *cis* and *trans* elements involved in the attenuation mechanism. We have shown that at least in some viral systems, RNA polymerase II can respond to a stem-loop structure in the RNA followed by polyuridine as a signal for the elongation block in vitro (1, 3, 19, 26, 37, 38, 40). In other systems, different sequences constitute the signal for the elongation block (2, 9, 39, 43, 48).

One of the systems in which transcription attenuation has been invoked is represented by the human immunodeficiency viruses (HIVs) (32, 41). Direct comparison between the HIV-1 and HIV-2 attenuator RNAs reveals that although they do not share sequence homology, both RNA species can assume extensive secondary structure. The RNA secondary structure seems to play a role both in activation of transcription initiation and in transcription attenuation (28, 42). Thus, the sequences between +19 and +42 in the RNA secondary structure of HIV-1, known as the *trans*-activation-responsive (TAR) element, are required for *trans* activation of transcription initiation by Tat (17, 18, 21, 41). Moreover, recent studies have suggested that TAR is a positive element which presents Tat to the transcription complex (10, 28, 41, 42). As far as transcription attenuation is concerned, Selby et al. (41) have suggested that the accumulated short transcripts (attenuator RNA) represent end products of RNA species that terminate within and beyond the HIV-1 LTR and are processed back to the stable RNA stem-loop. Recently, processing of longer RNA species to yield the TAR RNA stem-loop has also been observed in vitro (45). From these observations, it is not clear whether the TAR RNA stem-loop is an attenuation signal, a processing signal, or both.

Chamberlin and his colleagues have developed a system in which purified RNA polymerase II is able to transcribe defined dC-tailed DNA templates efficiently and specifically (12, 22). This system allows studies of elongation and termination on defined DNA sequences under conditions in which processing and nucleolytic cleavage of transcripts do not occur. Hence, this is an appropriate system for the definition of the sites, signals, and factors that mediate termination by RNA polymerase II. Using this experimental system, studies of intrinsic termination by RNA polymerase II (termination signals recognized by the polymerase protein itself in the absence of other protein factors [36, 44]) have shown that at least in the attenuators tested, RNA secondary structures are not required to stop elongation by the purified enzyme (13, 24, 36).

In this study, using the dC-tailed template system (22), we have found that transcription of the HIV-1 LTR by purified RNA polymerase II yields an attenuator RNA whose 3′ end maps to the end of the TAR stem-loop, approximately 60 to 65 nucleotides (nt) downstream of the in vivo initiation site. Furthermore, transcription attenuation occurs only under high-salt conditions which cause transcript displacement from the DNA template, thus allowing the RNA to fold into secondary structure. Evidence is provided that the purified RNA polymerase II recognizes RNA secondary structure as an attenuation signal. We suggest that Tat, alone or in combination with other factors, relieves the elongation block at the intrinsic attenuation signal.

MATERIALS AND METHODS

Plasmid constructions. The pGEM-TAR-CAT plasmid was constructed as follows. A *Sca*I-*Eco*RI fragment (575 bp)

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from the pTAR1-CAT (a gift of M. B. Peterlin) was inserted at the *Hin*cII-*Eco*RI sites of pGEM-1 (Promega) (Fig. 1C).

Plasmids 1 to 4 were constructed as follows. A *Pvu*II DNA fragment (350 bp) from pGEM-TAR-CAT was inserted at the *Eco*RV and *Hin*cII sites of pBluescript polylinker (Stratagene) in two orientations, generating the four clones (Fig. 3C).

Preparation of dC-tailed templates. The templates were linearized by restriction enzyme *Pst*I or *Kpn*I, both of which generate 3′ protruding ends, and tailed at both ends with dCTP by using terminal transferase (Boehringer Mannheim). Following ethanol precipitation, the dC-tailed DNAs were digested with a second restriction enzyme, and the selected tailed fragment was purified from agarose gel (22).

Purification of RNA polymerase II from HeLa cells. Two preparations of RNA polymerase II were used. One was purified by the procedure described by Hodo and Blatti (20); the second, a gift of D. Reinberg and Hua Lu, was purified as described previously (29). Similar results were obtained with the two preparations. Transcription factor IIF (TFIIF) was a gift of D. Reinberg and O. Flores. TFIIS was a gift of R. Weinmann. The transcription factors were used as previously described (6).

Transcription of 3′-tailed templates. Transcription reaction mixtures were incubated in 20 μl of transcription buffer containing 70 mM Tris-HCl (pH 7.9), 0.1 M NH_4Cl, 6 mM $MgCl_2$, 5 mM spermidine, 10% glycerol, 0.15 mM dithiothreitol, 0.6 μg of tailed template, and 1 μl of HeLa RNA polymerase II (2.8 U) at 30°C for 5 min. A pulse buffer containing, in 5 μl, 600 μM each ATP, CTP, and GTP, 20 μM UTP, and 30 μCi of [α-^{32}P]UTP was then added to the transcription reaction mixture and incubated for 1 min. Transcription elongation was then chased with 5 μl of chase buffer containing 600 μM UTP in the presence or absence of 0.3 M KCl or by diluting the reaction mixture with chase buffer containing 20 mM Tris-HCl (pH 7.9), 5 mM $MgCl_2$, 2 mM dithiothreitol, 4 mM creatine phosphate, and 700 μM each ATP, UTP, CTP, and GTP. The final volumes of the reaction mixtures are indicated in the figure legends. Similar results were obtained with or without diluting the chase reaction mixtures. No reinitiation of labeled transcripts can be observed under the chase conditions, because the high KCl concentration in the chase buffer prevents transcription initiation (data not shown). Transcription elongation was carried out for the indicated times and stopped by the addition of 2 volumes of stop buffer containing 20 mM EDTA, 0.2% sodium dodecyl sulfate, 200 μg of proteinase K per ml, and 30 μg of tRNA. Nucleic acids were collected by ethanol precipitation, resolved by electrophoresis on 6% (wt/vol) polyacrylamide–8.3 M urea gels, and visualized by autoradiography.

RESULTS

A block to transcription elongation by purified RNA polymerase II occurs within the LTR of HIV-1. In these in vitro transcription studies with purified RNA polymerase II (22), we used several dC-tailed DNA templates which contain the HIV-1 long terminal repeat (LTR) sequences from −138 to +185 relative to the in vivo transcription start site, followed by 250 bp from the chloramphenicol acetyltransferase (CAT) gene. The purified polymerase initiates transcription on these templates at discrete sites at the single-stranded tail double-stranded duplex junction (22). In all experiments, a pulse-chase protocol was used as follows. After a 1-min pulse with [α-^{32}P]UTP, the transcription reaction mixture

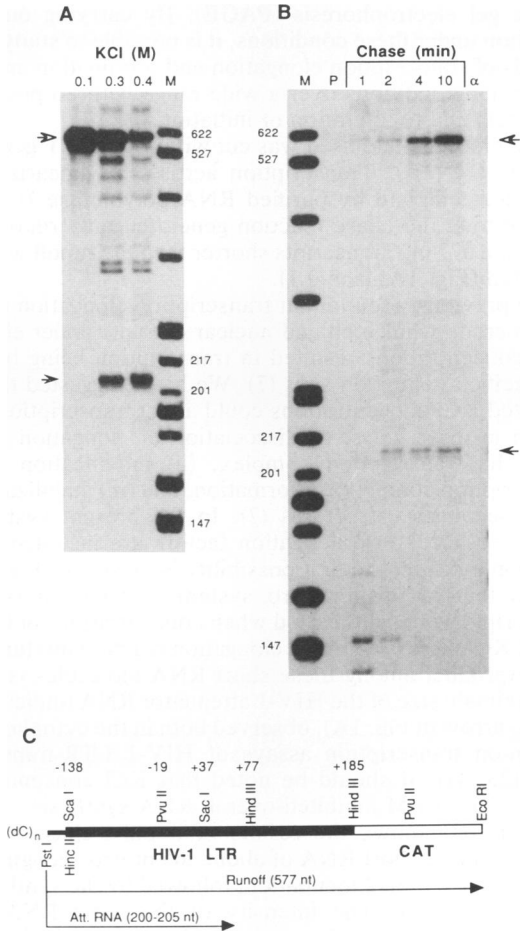

FIG. 1. Evidence that transcription elongation by purified RNA polymerase II is blocked within the HIV-1 LTR. A dC-tailed template of pGEM-TAR-CAT (see Materials and Methods) was transcribed in 25 μl under pulse-chase conditions (see Materials and Methods). (A) Pulse-chase experiment. Following a 1-min pulse with [α-^{32}P]UTP, the transcription reaction mixtures were diluted with 200 μl of chase buffer containing the indicated KCl concentrations and incubated for additional 30 min. The labeled RNA was purified and analyzed by PAGE as detailed in Materials and Methods. (B) Kinetics of transcription elongation under chase conditions containing 0.3 M KCl. Following a 1-min pulse as for panel A in a reaction mixture containing 100 μl, chase buffer was added to a final concentration of 0.3 M KCl. Samples were taken at the times indicated and placed in stop buffer as described in Materials and Methods. The labeled RNA was purified and analyzed by PAGE as detailed in Materials and Methods. In lane α, 1 μg of α-amanitin per ml was added to the reaction mixture prior to initiation. Lane P represents a reaction that was stopped after a 1-min pulse. Lanes M show size markers obtained by end labeling of the *Hpa*II DNA fragments of pBR322; sizes are indicated in nucleotides. The same size markers are used in all figures. (C) Schematic representation of the template used for transcription. HIV-1 LTR sequences are represented by the dark box, CAT sequences are shown by the open box, and the polylinker sequences are shown by a thick line. Numbers above the restriction enzymes indicate the locations relative to the in vivo transcription start site (+1). The expected lengths of the runoff RNA and attenuated (Att.) RNA are indicated.

was diluted with various KCl concentrations and transcription was allowed to proceed for an additional 30 min. The labeled RNA synthesized was then analyzed by polyacrylamide gel electrophoresis (PAGE). By carrying out transcription under these conditions, it is possible to study single rounds of transcription elongation and termination and vary elongation conditions over a wide range with no possibility of effects due to alteration of initiation.

The first template used was cut within the CAT gene with *Eco*RI (Fig. 1C). Transcription across this linearized, 3′-extended template by purified RNA polymerase II (0.1 M KCl) during the chase reaction generated only runoff transcripts of 577 nt. Transcripts shorter than the runoff were not detected (Fig. 1A, lane 0.1).

We previously found that transcription elongation carried out in crude whole cell and nuclear extracts under elevated KCl concentrations resulted in transcription being blocked at specific attenuation sites (7). We have suggested that the elevated KCl concentrations could affect transcription elongation in three ways: (i) dissociation of elongation factors from the transcription complex, (ii) modification of the transcription complex conformation, and (iii) stabilization of RNA secondary structures (7). In the present system, in which no accessory elongation factors are included in the reaction mixture, the first possibility is excluded. Figure 1A shows that in this in vitro system as well, short RNA transcripts were synthesized when concentrations of 0.3 and 0.4 M KCl were used in the elongation reaction mixture. The major product among these short RNA molecules is of the approximate size of the HIV-1 attenuator RNA (indicated by a solid arrow in Fig. 1A), observed both in the cytoplasm and in run-on transcription assays of HIV-1 LTR-transfected cells (23, 41). It should be noted that KCl concentrations higher than 0.4 M inhibited overall RNA synthesis.

Figure 1B shows the results of a pulse-chase kinetics experiment. A short RNA of about 205 nt was recognized at the first 1 to 2 min of incubation, followed by the synthesis of the runoff RNA. The intensity of the short RNA band remained almost constant upon prolonged incubation. The increase in the intensity of the runoff band appears to result from RNA molecules longer than the attenuator RNA which were further elongated during the chase (compare lanes 2 and 4 min). On the basis of the kinetics profile and the inclusion of only purified RNA polymerase II in this transcription system (the system is devoid of RNase activity; data not shown), we suggest that the short RNA molecules were products of a specific block to transcription elongation within the LTR and not of RNA processing. The sensitivity of the two major transcripts to α-amanitin (1 μg/ml) (Fig. 1B, lane α) indicates that these transcripts are indeed polymerase II specific.

The block to transcription elongation occurs under conditions that allow transcript displacement from the DNA template. In contrast to the in vivo situation, in which the nascent RNA is displaced from the template during elongation (11), in the dC-tailed template system the nascent RNA remains hybridized to the DNA template (12, 22). In experiments using the dC-tailed template to examine the role of RNA secondary structure in transcription regulation, it is essential to establish conditions that allow displacement of the nascent RNA during elongation and folding into secondary structure. We therefore first tried to verify whether the pattern of RNA synthesized at 0.3 and 0.4 M KCl, but not at 0.1 M KCl (Fig. 1A), correlates with displacement of the nascent RNA from the DNA template. Transcript displacement was determined by its sensitivity to digestion by RNase H (which digests hybridized RNA) and RNase A (which digests displaced RNA). The runoff synthesized at 0.1 M KCl was digested almost completely with RNase H (Fig. 2, lane 3) and only partially with RNase A (lane 4), indicating that most of the runoff transcripts remained fully hybridized to the DNA template. The runoff synthesized at 0.3 M KCl was also digested almost completely with RNase H (lane 7) and only partially digested with RNase A (lane 8), indicating that most of the RNA synthesized under these conditions also remained hybridized to the DNA template. In contrast, the attenuator RNA synthesized at 0.3 M KCl (indicated by the solid arrow) was resistant to RNase H digestion (lane 7) but sensitive to RNase A digestion (lane 8), indicating that it was mostly displaced from the DNA template. These results strongly suggest that transcript displacement from the DNA template is an obligatory condition for the production of the attenuator RNA. It is interesting that elevated concentrations of monovalent cation have been shown to increase the termination efficiency of *Escherichia coli* RNA polymerase

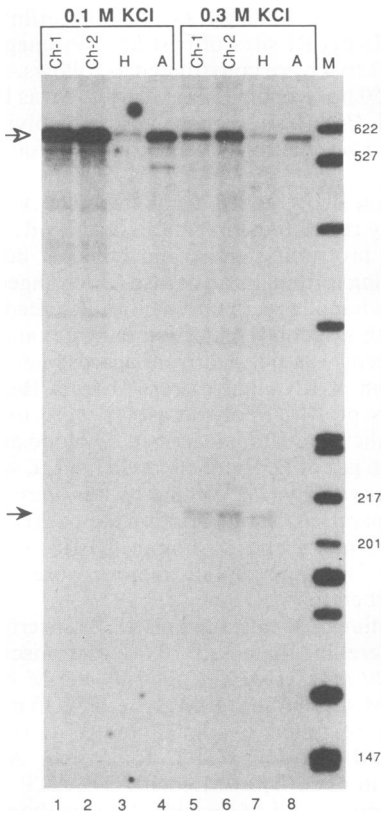

FIG. 2. Displacement of the attenuated transcripts from the DNA template. Transcription under pulse-chase conditions was performed as described in Materials and Methods. The 20-min chase reaction was carried out in 0.1 M KCl (lanes 1 to 4) or in 0.3 M KCl (lanes 4 to 8). The chase was carried out in 100 μl. Following the 20-min chase, all reaction mixtures were brought to 0.1 M KCl in a final volume of 300 μl and incubated at 30°C for a further 60 min, after which stop buffer was added. At the beginning of the 60-min period, RNase H (2 U; Boehringer Mannheim) (lanes H) and RNase A (20 μg/ml; Worthington) (lanes A) were added to the indicated reaction mixtures. In lanes Ch-1, transcription was stopped after the 20-min chase. In lanes Ch-2, reaction mixtures were incubated for an additional 60 min. The labeled RNA was purified and analyzed by PAGE as detailed in Materials and Methods.

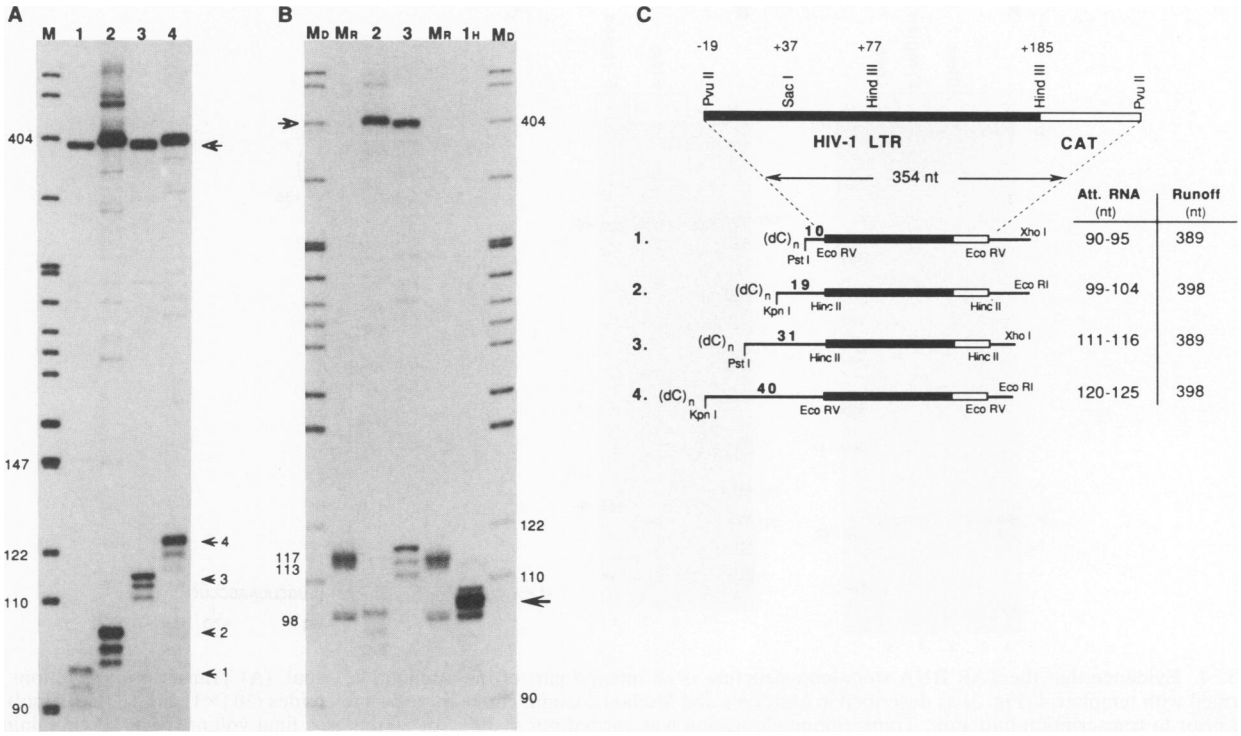

FIG. 3. Mapping of the attenuated HIV-1 RNA. Transcription under pulse-chase conditions by purified RNA polymerase II was carried out on four dC-tailed templates. The final volumes of the chase reactions were 100 μl, and the KCl concentration 0.3 M. In panel A, lanes 1 to 4 represent the RNA profiles synthesized on each of the four templates shown in panel C. The open arrows point to the runoff RNAs; the solid arrows point to the attenuated transcripts of constructs 1 to 4. In panel B, the reaction products shown in lanes 2 and 3 in panel A were run in lanes 2 and 3. In lane 1H, transcription was carried out on a template 1 (see panel C) that was dC tailed at the *Pst*I site and then digested by *Hin*dIII (cut at +77). The purified *Pst*I-*Hin*dIII fragment was transcribed under pulse-chase conditions at 0.1 M KCl. The runoff obtained is indicated by the arrow. Lane MR shows RNA size markers of 98, 113, and 117 nt. Lane MD shows DNA size markers. (C) Schematic representation of the four constructs used in the transcription reactions of panel A. For details of their construction, see Materials and Methods. Briefly, the TAR element (*Pvu*II DNA fragment) was inserted at four positions within the Bluescript plasmid polylinker, creating four templates that contain the TAR element downstream of several polylinker sequences. Each template was digested by *Pst*I or *Kpn*I and then dC tailed by terminal transferase. The four templates thus differ by the lengths of the polylinker sequences located upstream of the TAR sequences. The four templates are designated 1 to 4. HIV-1 sequences are represented by the filled-in box, CAT sequences are indicated by the open box, and the Bluescript polylinker is represented by a line. The expected (calculated) attenuated (Att.) and runoff transcripts of each of the templates are shown in the table (assuming that transcription initiates at the dC tail double-stranded junction and attenuates 55 to 60 nt downstream of the in vivo start site).

at some termination sites (33) and also of the purified mammalian enzyme (13).

Mapping of the attenuator RNA. Mapping of the attenuator RNA by the conventional methods of S1 and RNase A-RNase T_1 protection yielded several bands, probably because of its stable secondary structure (data not shown). We have therefore used the following alternative approach for mapping the attenuator RNA. The *Pvu*II DNA fragment of the HIV-1 plasmid pTAR1-CAT (spanning residues −19 to +185 of the HIV-1 LTR and 150 bp of the CAT gene; the transcription start site is taken as +1) was cloned in two orientations at the *Eco*RV and *Hin*cII sites within the Bluescript plasmid polylinker. Each of the resulting four clones was digested with either *Kpn*I or *Pst*I and dC tailed (22). Thus, four templates containing the TAR element, which differ in the length of the 5′ Bluescript polylinker (of the sense strand), were obtained (Fig. 3C). If, in the four templates, transcription initiates at the same dC residue of the tail and attenuation occurs at the same site, the lengths of the attenuator RNAs will differ by the lengths of the Bluescript polylinker only. The lengths of the extended 3′ ends (of the template strand) and the expected (calculated) lengths of the attenuator (23) and runoff RNAs are presented in Fig. 3C.

When transcription elongation was carried out on the four tailed templates, both an attenuator and runoff transcripts were synthesized (solid and open arrows, respectively, in Fig. 3A). The attenuator RNA appears in three bands. In each panel, the difference in length between the long and short attenuator RNAs is 5 to 10 nt. If transcription initiates at the single-stranded and double-stranded junction, then the 3′ ends of these attenuator RNAs map to residues 57 to 67 downstream of the in vivo transcription start site. This is about 2 to 7 nt longer than the estimated length of 55 to 60 nt of the in vivo attenuator RNA (23). It is possible, however, that the apparent small difference in length results from the fact that in vitro transcription initiates not at the single double-stranded junction but at a site located a few nucleotides upstream (13). Alternatively, it is possible that in vivo there is nibbling of a few nucleotides at the 3′ end of the attenuator RNA. For determining the transcription initiation site, construct 1 in Fig. 3C was digested with *Hin*dIII

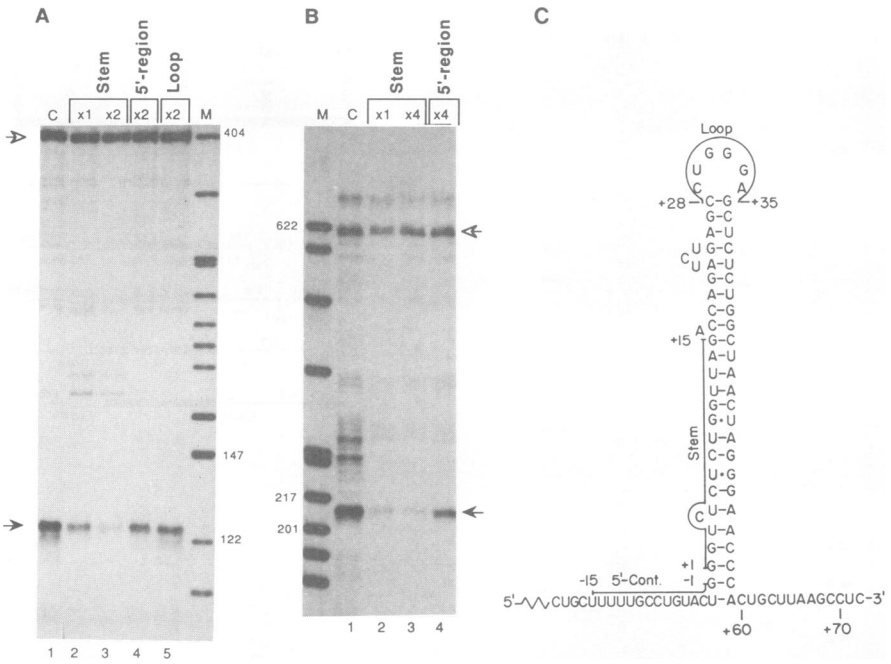

FIG. 4. Evidence that the TAR RNA stem-loop structure is an integral part of the attenuation signal. (A) Transcription reactions were performed with template 4 (Fig. 3) as described in Materials and Methods. Complementary oligonucleotides (30 [×1] and 60 [×2] pmol) were added prior to transcription initiation. Transcription elongation was carried out at 30°C for 20 min in a final volume of 30 μl containing 0.3 M KCl. Lane C shows a control in which no oligonucleotide was added prior to transcription initiation. (B) Transcription reactions were performed with the pGEM-TAR-CAT tailed template shown in Fig. 1C. Pulse-chase transcription was carried out as described in Materials and Methods except that the transcription reaction was carried out at 21°C. Oligonucleotides (30 [×1] and 120 [×4] pmol) were added to the chase mixture in a final volume of 35 μl. Lane C shows a control in which no oligonucleotide was added to the chase buffer. The labeled RNA was purified and analyzed by PAGE as detailed in Materials and Methods.

(residue 77) in order to allow the synthesis of a short runoff transcript, thus enabling a better estimation of its length. If transcription initiates at the single double-stranded junction, the expected length is 107 nt. If transcription initiates at an upstream residue, a longer runoff will be synthesized. The synthesized runoff was then analyzed by denaturing gel electrophoresis using DNA (Fig. 3B, lane MD) and RNA (lane MR) fragments as size markers. The attenuator RNAs synthesized on constructs 2 and 3 were also included in the gel electrophoresis. The length of the major runoff band is about 107 nt (lane 1H), indicating that the major transcription initiation site is at the single double-stranded junction and that attenuation occurs heterogeneously 57 to 67 nt downstream of the in vivo transcription start site. The attenuator RNA synthesized in the in vitro system is therefore longer by a few nucleotides than the in vivo attenuator RNA (23, 41).

The stem-loop structure of the TAR element is an integral part of the attenuation signal. The finding that transcript displacement from the DNA template correlates with the observed block to transcription elongation at the HIV-1 TAR region suggested to us that in vitro, purified RNA polymerase II recognizes the TAR RNA secondary structure as a signal for the block to transcription elongation. To test this possibility, we used an approach that was successfully exploited by Yanofsky et al. in their studies on the *trp* attenuator (15, 46) and subsequently by us in our studies on the simian virus 40 attenuator (25). We synthesized three oligonucleotides: one complementary to the left side of the stem, one complementary to transcript sequences upstream to the stem, and one complementary to the sequences in the loop (Fig. 4C). The addition of the oligonucleotide complementary to the stem during transcription is expected to interfere with folding of the RNA into the predicted secondary structure and consequently decrease the elongation block at the attenuation site. The addition of the other two oligonucleotides should not interfere, or interfere only slightly, with the formation of the RNA secondary structure, and they served as controls.

Two experiments were performed. In the first, the complementary oligonucleotides were added prior to transcription initiation and the whole reaction was carried out at the standard temperature of 30°C. Following a preliminary titration reaction to determine the minimal concentration of the oligonucleotide which would prevent the elongation block, a subsequent experiment demonstrated that the addition of 30 to 60 pmol had this effect. Addition of the oligonucleotide complementary to the stem reduced the transcriptional block at the attenuation site in a dose-dependent manner (Fig. 4A). With the addition of 30 pmol the reduction was 60%, while with the addition of 60 pmol the reduction was more than 90% (lanes 2 and 3). Competition with the oligonucleotides complementary to either the 5′ region or the loop was significantly less effective in reducing the elongation block (lanes 4 and 5). No RNase H activity was detected in our in vitro transcription system, indicating that the reduction in the amount of the attenuator RNA was not due to its degradation by RNase H (data not shown). The small decrease seen in overall RNA synthesis was probably due to binding of a fraction of the RNA polymerase molecules to the oligonucleotides present in the transcription buffer. In the second experiment, we used the template shown in Fig.

1C. In this experiment, the complementary oligonucleotides were added following the 1-min pulse, together with the elongation chase buffer. We had previously found no effect when the elongation reaction was carried out at the standard temperature of 30°C (data not shown), probably because the high elongation rate prevented the hybridization of the oligonucleotide to its complementary sequences in the attenuator RNA (25). To allow better conditions for hybridization, the chase reactions were carried out at 21°C, a temperature at which the elongation rate is greatly reduced (25). At this temperature, the oligonucleotide complementary to the stem significantly decreased synthesis of the attenuator RNA at both concentrations (Fig. 4B, lanes 2 and 3), whereas the oligonucleotide complementary to the 5′ region had only a slight effect (lane 4). The slight effect on synthesis of the attenuated transcripts seen with the control oligonucleotides may result from a partial interference with the formation of the RNA secondary structure. We therefore conclude that RNA secondary structure containing the TAR element is involved in directing the elongation block. In addition, since the competition effect is seen only during the slow, and not during the fast, synthesis of the nascent RNA, this result excludes the possibility that the attenuator RNA is processed from the runoff RNA.

Transcription elongation by purified RNA polymerase II is blocked at a synthetic RNA stem-loop structure. To determine whether a specific sequence in the TAR stem-loop element is needed for the block to elongation by purified RNA polymerase II, or whether the purified enzyme can respond to any stem-loop structure independent of specific sequence, we analyzed the in vitro transcription of a template which includes an insert of a chemically synthesized sequence encoding a stable RNA secondary structure of −48 kcal (ca. −200 kJ) followed by only three consecutive uridines (plasmid −48 Kcal-3T; 5) (Fig. 5B). Transcription of this construct in the whole cell extract transcription system in the presence of Sarkosyl leads to only a brief and insignificant pause at a site located at the stem-loop structure (5). Transcription of this template with purified RNA polymerase II at increasing KCl concentrations leads to a corresponding increase in the production of attenuator RNA (Fig. 5A). We therefore conclude that it is the stability of the RNA stem-loop structure and not the specific sequence of the stem-loop element that is the signal for a block to transcription elongation.

The block to elongation at the HIV-1 attenuation site is irreversible. Two experiments were performed to characterize the nature of the elongation block at the HIV-1 attenuation site. In the first, transcription elongation reactions were carried out at 0.3 M KCl for 20 min. At this time a fraction of the polymerase molecules are blocked at the attenuation site, giving rise to the synthesis of the attenuator RNA (Fig. 1). The reaction mixtures were then diluted to the final KCl concentrations indicated in Fig. 6A and incubated for an additional 20 min. If the elongation block is due to pausing of the polymerase at the attenuation site, then dilution of the elongation reaction mixture to the KCl concentrations at which there is no elongation block (0.07 to 0.15 M; Fig. 1A) should allow elongation to resume. As demonstrated in Fig. 6A, there was a small decrease in the intensity of the attenuated RNA band upon dilution from 0.3 to 0.2 M KCl; after that, there were not visible differences among the various KCl concentrations.

TFIIS and TFIIF are capable of preventing elongation blocks at attenuation sites (6, 34, 35). Moreover, TFIIS is also capable of releasing the polymerase which pauses at attenuation sites (6, 44). In the second experiment, we tested whether these two general elongation factors are capable of releasing the elongation block at the HIV-1 attenuation site. After 20 min of elongation in the presence of 0.3 M KCl, the reaction mixture was diluted to 0.1 M KCl, (a concentration in which both transcription factors are fully active [6]) and the factors were added. The results of Fig. 6B show that the inclusion of saturating amounts of these elongation factors did not release the elongation block. Furthermore, in this experiment no decrease in the intensity of the attenuated transcripts was recognizable upon diluting the KCl concentration. On the basis of the results of Fig. 6, we suggest that

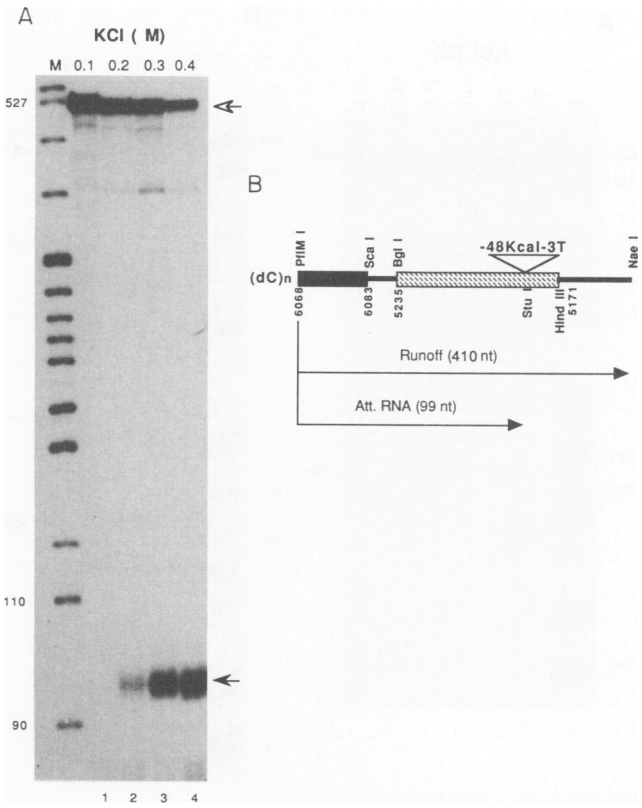

FIG. 5. Evidence that transcription elongation by purified RNA polymerase II is blocked at a synthetic stem-loop structure. (A) Construction of the −48 Kcal/3T plasmid (5) is described in Materials and Methods. The template was tailed at the *Pfl*MI site (residue 6068 of adenovirus type 2) and digested again at the unique *Nae*I site of pGEM-1. The pulse-chase transcription reaction was carried out as described in Materials and Methods. The indicated KCl concentrations were added to the chase reaction mixtures in 200 μl. Elongation was allowed to proceed for 30 min at 30°C. The labeled RNA was purified and analyzed by PAGE as detailed in Materials and Methods. (B) Schematic representation of the tailed template used for panel A. The lengths of the runoff RNA and attenuator (Att.) RNA are indicated. The adenovirus type 2 DNA fragment (residues 6068 to 6083) is shown in the black box, and the simian virus 40 DNA fragment (residues 5235 to 5171) is shown in the hatched box. The position where the synthetic DNA fragment was inserted is indicated (−48 Kcal-3T). Note that the runoff of 410 nt runs with a DNA marker of about 500 nt. This 20% discrepancy may occur in the upper part of the 6% polyacrylamide-urea gel used in this experiment, which is adequate for sizing fragments of 60 to 250 nt. In sizing fragments of 60 to 250 nt, the RNA could sometimes be about 5% smaller than estimated from the DNA markers.

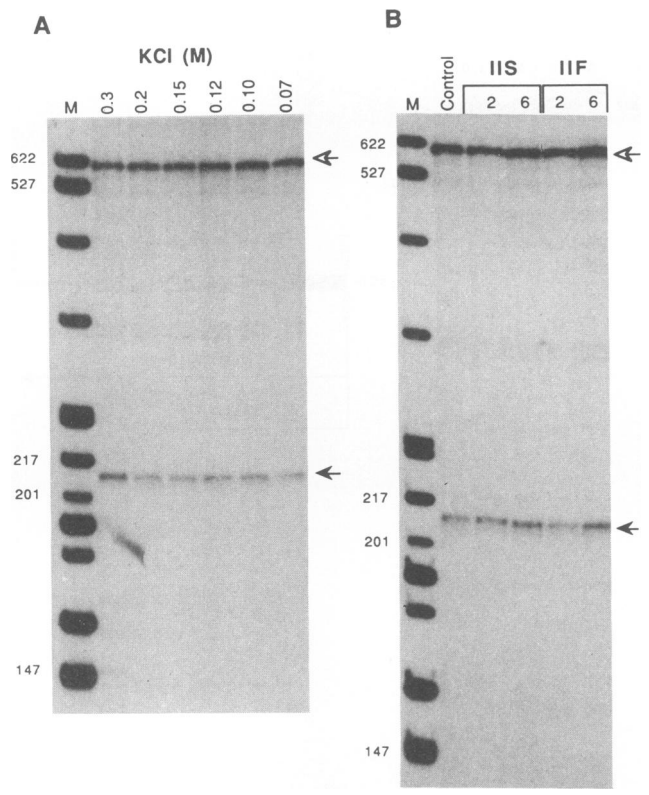

FIG. 6. Irreversibility of the block to elongation at the HIV-1 attenuation site. The pGEM-TAR-CAT construct was dC tailed (see Fig. 1C) and transcribed under the following conditions. (A) After pulse-chase transcription for 20 min at 0.3 M KCl in a final volume of 100 μl, the reaction mixtures were diluted, adjusted to the indicated final KCl concentrations, and incubated for an additional 20 min at 30°C. (B) After pulse-chase transcription in 30 μl for 20 min at 0.3 M KCl, the reaction mixtures were diluted to 0.1 M KCl (final volume, 90 μl), 2 and 6 μl each the TFIIF and TFIIS (6) were added, and the mixtures were incubated for an additional 20 min. These amounts of transcription factors were previously found to relieve the elongation block at the adenovirus type 2 attenuation site (6). In the control lane, no factor was added with the dilution. The labeled RNA was purified and analyzed by PAGE as detailed in Materials and Methods.

the majority of the RNA polymerase molecules are either irreversibly blocked or released at the HIV-1 attenuation site. It is noteworthy that a true transcription termination occurs at the TII site of the c-*myc* attenuator when it is transcribed in the dC-tailed template system (24).

DISCUSSION

In this study, we have used an in vitro transcription system that allows efficient transcription initiation by purified RNA polymerase II in the absence of accessory factors (22). This system permits the study of transcription elongation separately from the process of promoter-directed initiation. The results demonstrate that the production of the HIV-1 attenuator RNA results from a block to transcription elongation and not from RNA processing and that the block is due to an intrinsic property of RNA polymerase II in response to a transcribed stable stem-loop structure.

These studies thus support the notion that the attenuator RNAs observed in run-on transcription experiments and in isolated nuclei and cytoplasm of HIV-1-transfected cells in the absence of Tat protein (23, 41) arise, at least in part, from premature transcription termination. It is still possible, however, that part of the attenuator RNA molecules observed in vivo result from processing of transcripts longer than the attenuator RNA, yielding an attenuator RNA having a stem-loop structure which is resistant to further nucleolytic cleavage. This is supported by the observation that the in vitro-synthesized attenuator RNA seems to be a few nucleotides longer than the in vivo attenuator RNA (23). This difference is probably due to nibbling of a few nucleotides, up to the end of the stem-loop, which occurs in vivo.

The attenuator RNA was synthesized in the DNA tailed-template system in high (0.3 to 0.4 M) but not in low (0.1 M) KCl concentrations. We found that when transcription was carried out in 0.1 M KCl, only runoff transcripts were synthesized and they were hybridized to the template strand. However, when transcription was carried out under high KCl concentrations (0.3 to 0.4 M), while most of the runoff RNA was still hybridized to the template strand, the attenuator RNA was completely displaced from the template strand. Displacement of the attenuator RNA allows it to fold into secondary structure. These results therefore suggested to us that in vitro, purified RNA polymerase II recognizes the TAR RNA stem-loop structure as a signal for a block to transcription elongation. Indeed, when an oligonucleotide that can hybridize to the left arm of the stem structure was included in the elongation reaction mixture and therefore could interfere with the folding of the RNA into the predicted secondary structure (15, 25), the elongation block at the attenuation site was significantly decreased. We therefore conclude that RNA polymerase II is blocked within the TAR region in response to the secondary structure of the transcribed and displaced nascent RNA. The observation that a block to elongation occurs also in the presence of a synthetic stem-loop structure indicates that the polymerase responds to the stem-loop structure per se and not to a specific sequence in the RNA secondary structure. In this respect, it is not surprising that the TAR element of HIV-1 and HIV-2, although not sharing sequence homology, can nevertheless be folded into RNA secondary structures of similar stabilities (14) and therefore can function as a transcription attenuator.

The two attenuators analyzed in this study have relatively stable RNA secondary structures (free energies of folding for the structures are −37.6 and −48 kcal [ca. −157 and −200 kJ]/mol for the HIV-1 [32] and the synthetic [5] attenuators, respectively). The extent of the elongation block seen with the HIV-1 attenuator ranged between 10 and 50%, while that seen with the more stable synthetic attenuator exceeded 60%. It has previously been shown (13) that only 9% of the mammalian polymerase molecules were stopped at the *E. coli trp* attenuator (−22 kcal [ca. −92 kJ]/mol [27]) at low salt concentrations and 31% were stopped at 0.3 M NaCl. It should be mentioned, however, that the *trp* attenuator contains, in addition to the RNA secondary structure element, a U stretch following it that also contributes to an elongation block (24, 36, 44). The results presented here seem to be in agreement with our previous conclusion that there is a direct correlation between the stability of RNA secondary structure and the extent of the elongation block (5).

Although the KCl concentration in the microenvironment of the transcription complex is unknown, the concentration of KCl (0.3 M) in which the attenuator RNA was synthesized in vitro is above the physiological KCl concentration in the

cell. However, in vivo, the nascent RNA is displaced from the template (11) and can readily be folded into secondary structure. In addition, specific factors can modulate the formation of RNA secondary structure in vivo, as expected for a conditional block to transcription elongation. In the present in vitro system, the elevated KCl concentration favors, presumably, the formation of an RNA-RNA duplex over an RNA-DNA hybrid and/or changes the polymerase conformation to that which recognizes stem-loops as attenuators or terminators.

It is interesting that our efforts to identify the HIV-1 attenuator RNA in vitro by using conventional whole cell or nuclear extracts in the presence of either Sarkosyl (26, 38, 40) or high KCl concentrations (5, 6) were unsuccessful. The presence of either Sarkosyl or elevated KCl concentrations during transcription elongation was shown to remove elongation factors from the transcription complex, thus enabling the polymerase to respond to attenuation signals (5, 6, 7). It therefore appears that the RNA polymerase II associated with the elongation complex, although stripped of elongation factors, has properties different from those of the purified polymerase. Alternatively, it is still possible that additional elongation factors which are not removed from the elongation complex by either Sarkosyl or high KCl concentrations prevent the elongation block when whole cell or nuclear extracts are used as the transcription systems. It is also noteworthy that the HIV-1 attenuation signal has no similarities to other known prokaryotic or eukaryotic attenuators. Although the HIV-1 attenuator contains an RNA stem-loop structure like the rho-independent attenuator (47) and viral attenuators (1, 3, 19, 38), it is not followed by a stretch of uridylic acids residues.

In vivo, the TAR element is recognized as an RNA stem-loop structure by the Tat *trans*-acting protein (10) and by a P68 cellular nuclear protein (30). We suggest that this simple transcription system can be useful for studying the mechanism by which Tat, P68, or additional proteins regulate transcription elongation through the HIV-1 TAR region.

ACKNOWLEDGMENTS

We thank D. Reinberg and O. Flores for the gift of homogeneous TFIIF, R. Weinmann for TFIIS, D. Reinberg and Hua Lu for the highly purified RNA polymerase II, and A. Usheva for preparing RNA polymerase II. We also thank M. B. Peterlin for providing the pTAR1-CAT construct and Michael Walker for helpful discussions and comments.

This work was supported by grants from the National Institutes of Health (CA 14995); the United States-Israel Binational Science Foundation; the Israel Academy of Sciences and Humanities; the Minerva Foundation, Munich, Germany; and the Leo Forchheimer Center for Molecular Genetics.

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