The HIV-1 Inducer of Short Transcripts Activates the Synthesis of 5,6-Dichloro-1-β-d-benzimidazole-resistant Short Transcripts in Vitro*

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The HIV-1 inducer of short transcripts (IST) is an unusual promoter element that activates the synthesis of short transcripts from the HIV-1 promoter as well as from heterologous promoters. While the DNA sequences constituting IST have been characterized in some detail, little is known about the biochemical mechanisms underlying IST activity. Here, we describe a cell-free transcription assay that faithfully reproduces the synthesis of IST-dependent HIV-1 short transcripts. As in vivo, formation of these short transcripts requires a functional IST element and is repressed in the presence of the viral trans-activator Tat. Short transcript and full-length transcript synthesis respond differentially to variations in several reaction parameters, suggesting that the short and full-length transcripts are synthesized by transcription complexes with distinct biochemical properties. In particular, short transcript synthesis is resistant to the action of 5,6-dichloro-1-β-d-benzimidazole, an inhibitor of transcript elongation. Formation of transcription complexes directed by the IST element may, therefore, not require the activity of a factor inhibited by 5,6-dichloro-1-β-d-benzimidazole, such as the TFIIH-associated or pTEFb kinases.

EXPERIMENTAL PROCEDURES

Plasmids—Plasmid pU3RIII/pUC119 (generously provided by M. Laspia, Dartmouth Medical School) contains HIV-1 sequences from -642 to +82 fused to the chloramphenicol acetyltransferase gene and is identical to pU3RIII (7) except that the vector backbone is derived from pUC119. The plasmids used in the cell-free transcription assays, pHIV-1R/ML and derivatives, were constructed as follows. In the first step, the construct pU3RIII/pUC119, which contains two HindIII sites, was subjected to a partial digestion with HindIII, the ends were filled-in with the Klenow fragment of Escherichia coli DNA polymerase I, linear molecules were isolated on a gel and religated. A clone containing a single HindIII site at position +78 relative to the HIV-1 transcription start site was isolated and named pU3RIII/pUC119/HindIII. In the second step, the construct pU3RIII/pUC119/HindIII, which contains two SalI sites and a unique HindIII site, was subjected to partial digestion with SalI, followed by complete digestion with HindIII and phosphatase treatment. Linear molecules cleaved at the SalI site at position +141 relative to the HIV-1 transcription start site and at the HindIII site at position +78 relative to the HIV-1 transcription start site were isolated. This vector was ligated with a fragment generated by polymerase chain reaction from the pHIV-1/R template (4) and extending from the SalI site at position +141 to the HindIII site at position +78. The resulting construct, pHIV-1/R/ML, was similar to the pU3RIII/pUC119 construct, except that the HIV-1 sequences contained an engineered XhoI site at position -10, and a point mutation at position +77 that disrupts the polyadenylation signal. This construct contains unique XhoI and A/III sites at positions -10 and +64, respectively, relative to the HIV-1 transcription start site. The derivatives of pHIV-1/R/ML were generated by replacing the XhoI/A fragment with 5,6-dichloro-1-β-d-benzimidazole; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PIPES, 1,4-piperazinediethanesulfonic acid; nt, nucleotide.

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extending from −10 to +64 by the corresponding XhoI-AflII fragments derived from the various pHIV-1/R derivatives.

Template plasmids for the synthesis of antisense riboprobes were constructed as follows. pHIV-1/R was digested with HindIII, which cleaves just proximal to a bacteriophage T3 promoter oriented in an antisense direction relative to the HIV-1 promoter. Plasmid templates for the synthesis of antisense riboprobes were constructed as follows. Template plasmids pHIV-1/R/ML or ABC/ML were cleaved with EcoRI at position +329 in the chloramphenicol acetyltransferase coding sequences and the ends were filled in with the Klenow fragment of DNA polymerase I. Initial digestion with XhoI liberated a 334-base pair fragment which was ligated into the XhoI/HindIII cut pHIV-1/R described above, resulting in plasmids pHIV-1/ML/T3/ΔEco and pABC/ML/T3/ΔEco. Fig. IB depicts maps of the above constructs and the position of the antisense RNA probe with respect to the HIV-1 transcription unit.

**Cell-free Transcription Assay—**Plasmid templates were linearized at the NcoI site at position +630 with respect to the HIV-1 transcription start site, phenol and phenol/chloroform extracted, ethanol precipitated, and dissolved in deionized sterile water at a concentration of 250 ng/μl. Unless otherwise indicated, transcription reactions (20 μl) contained 25 ng of linearized plasmid, 7.5 mM MgCl₂, 50 mM KCl, 2 mM dithiothreitol, 10 mM HEPES-KOH (pH 7.9), 3.75 mM creatine phosphate, 10 mM dGTP, 7.5 mM ATP, 10 μM [α-32P]UTP (1 μl of 3000 Ci/mmol) in 50 mM Tricine, pH 7.6, NEN Research Products), and 10 μl of HeLa cell nuclear extract (80–120 μg of protein). Reactions were incubated at 30 °C for 60–75 min with no preincubation or presynthesis. Transcription was terminated by the addition of 200 μl of stop buffer (330 mM NaCl, 0.5% SDS, 10 mM Tris-HCl (pH 7.5), 10 μg/100 μl of total yeast RNA, 50 μg/100 μl of proteinase K), and, after a further incubation of 10 min at room temperature, nucleic acids were isolated by phenol/chloroform extraction and ethanol precipitation. Precipitated nucleic acids were then redissolved in formamide loading buffer, resolved by electrophoresis on denaturing 6% polyacrylamide gels, and visualized by autoradiography.

**RNA Stability Assay—**Plasmids pET7/R and pET7/ABC contain sequences from +1 to +82 of either pHIV-1/R or msABC (4), respectively, cloned downstream of the bacteriophage T7 promoter into the StuI site of plasmid pET7 (9). Internally labeled short transcripts carrying wild-type HIV-1 sequences from −1 to +68 or the same sequence containing the ABC mutations were synthesized as follows. The template plasmids were linearized at the AflII site at position +64 of the HIV-1 insert, phenol/chloroform extracted, ethanol precipitated, and redissolved in sterile deionized water at a concentration of 250 ng/μl. 250 ng of template were then used in a standard bacteriophage T7 RNA polymerase (Boehringer Mannheim) in vitro RNA synthesis reaction according to the recommendations of the manufacturer, except that the reaction contained 6.25 μM [α-32P]UTP (NEN Research Products, 800 Ci/mmol) and 100 μM unlabeled CTP. The resulting transcripts were purified as described above, quantitated by a scintillation counter, and redissolved in sterile deionized water at a concentration of 20,000 counts/μl. 40,000 counts of radiolabeled wild-type or ABC short transcripts were then added to reactions that were identical to a standard cell-free transcription reaction except that they did not contain [α-32P]UTP.

**RESULTS**

**Synthesis of IST-dependent Short Transcripts in a Cell-free Transcription System**—We have previously characterized mutations within the HIV-1 promoter region that affect preferentially the synthesis of short transcripts, full-length transcripts, or both (4). To determine whether we could obtain short transcript synthesis in vitro, we compared the RNA products generated by a battery of such mutants in a cell-free system. However, we detected only weak transcription signals when a HeLa cell nuclear extract was programmed with the constructs we had used before in our in vitro studies (pHIV-1/R and its mutant derivatives, data not shown). We therefore subcloned HIV-1 sequences containing the wild-type or mutant IST sequences (from −141 to +78) into an HIV-1 construct that had been used successfully for in vitro transcription studies (7), thus creating the construct pHIV-1/R/ML and its derivatives, whose sequences in the −30 to +92 region are shown in Fig. 1A. These constructs contain HIV-1 sequences from −642 to +82 relative to the transcription start site fused to the chloramphenicol acetyltransferase coding sequences. The polyadenylation signal starting at position +74, which is not used in the 5′-long terminal repeat in vivo, was inactivated by a point mutation at position +77 (underlined in Fig. 1A) to avoid the possibility of artificial polyadenylation of transcripts in vitro. A schematic of pHIV-1/R/ML is depicted in Fig. 1B.

The pHIV-1/R/ML plasmid and mutant derivatives were linearized with NcoI at position +630 downstream of the HIV-1 transcription start site, and were then used to program a HeLa cell nuclear extract in the presence of radiolabeled uridine triphosphate. The RNA products were fractionated on a sequencing gel, and the results are shown in Fig. 2. As typically observed with this type of assay, a number of background bands resulting from transcription initiation at cryptic promoters, within vector sequences and from end labeling of endogenous RNAs found in the nuclear extract were detected (e.g., bands around the 110 and 67 nucleotide markers). In addition, we detected two classes of specific transcripts: transcripts of about 630 nt, the length expected for full-length run-off transcripts, and short transcripts of about 83 and 85 nt in length (Fig. 2, lane 3, bands labeled FL and ST, respectively). Note that only the sections of the gel containing the bands of interest are
**HIV-1 Short Transcripts in Vitro**

**A**

![Diagram of HIV-1 Short Transcripts](image)

**B**

![Diagram of HIV-1 Templates and Probes](image)

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The ability of each mutant to synthesize short transcripts in a transfection assay (3, 4) is indicated on the right (+, no selective defect in short transcript synthesis; −, selective defect in short transcript synthesis). Figure 1B, schematic of the wild-type template plasmid used in the cell-free transcription assay (pHIV-1/R/ML), the template plasmid used for in vitro synthesis of the wild-type antisense riboprobe (pHIV-1/ML/T3/ΔEco), the location of the antisense riboprobe with respect to the transcription unit in pHIV-1/R/ML, and the protected transcripts. The arrow in pHIV-1/R/ML denotes the start site of transcription. The arrow in pHIV-1/ML/T3/ΔEco refers to the bacteriophage T3 promoter in antisense orientation used to synthesize the antisense riboprobe.

shown; a full gel is shown in Fig. 5A.) The appearance of these transcripts depended on the presence of an intact HIV-1 TATA box, because they were not present when the TATA4/ML construct, in which the HIV-1 TATA box is debilitated by point mutations (see Fig. 1A), was used as a template (data not shown but see Fig. 7, lanes 1 and 2). This suggests that these two classes of transcripts correspond to RNAs correctly initiated at the HIV-1 promoter.

To determine whether the 83- and 85-nt long transcripts corresponded to IST-dependent short transcripts, we transcribed several promoter constructs carrying mutations that corresponded to IST-dependent short transcripts, we transcribed at the HIV-1 promoter.

In vitro, the short transcript doublet was present with mutants ms5/ML, ms4/ML, ms8/ML, and ms2/ML (Fig. 2, lanes 4–7), none of which exhibit selective deficits in short transcript synthesis in vivo, but not with mutants ms1/ML, pIst/ML, msABC/ML, and msAB/ML, which do not direct the formation of short transcripts in vivo (lanes 1, 2, 9, and 10). Significantly, the msABC/ML mutant, which directs low but detectable levels of short transcripts in vitro (lane 11). Furthermore, the pIst/ML construct, in which 11 base pairs of transcribed sequence between the two IST half-elements are deleted (Fig. 1A) and which directs the formation of correspondingly shorter short transcripts in vitro (lane 12, bands labeled sat). The different abilities of these constructs to direct the formation of short transcripts did not reflect a general defect in transcription, because all constructs directed the synthesis of significant levels of full-length transcripts (lanes 1–12, band labeled FL). Thus, the extensive correlation between the ability of the various mutants to support the synthesis of short transcripts in vivo and the synthesis of the 83–85-nucleotide doublet in vitro suggests that synthesis of this doublet reflects the activity of the IST element.

To allow for efficient internal labeling of the transcripts, the cell-free transcription assay contained a much lower concentration of UTP (7.5 μM unlabeled, 165 nm radiolabeled) than of the other three nucleotides (250 μM each). Thus, it was necessary to ascertain that the short transcripts did not result from artificial pausing or termination by the RNA polymerase due to a rate-limiting UTP concentration. We therefore varied the concentration of UTP in the transcription reaction from 1.0 to 250 μM by increasing the amounts of non-radiolabeled UTP, and determined the amounts of short transcripts and full-length transcripts generated by the wild-type template pHIV-1/R/ML at each UTP concentration. Fig. 3A shows the results of this experiment, and Fig. 3B the percentage of maximum amounts of short and full-length transcripts obtained at each UTP concentration.

As shown in Fig. 3A, the radiolabeled signal corresponding to both short and long transcripts decreased as the ratio of unlabeled UTP to radiolabeled UTP ([UTP]/[UTP], see top of figure) increased, as expected. However, as shown in Fig. 3B, actual synthesis of both the short and full-length transcripts...
increased steadily as a function of total UTP concentration and approached saturation at 250 μM UTP, as determined after quantitation of the signals with a PhosphorImager and correction for the increase in the unlabeled to radiolabeled UTP ratio. Remarkably, the synthesis curves of short and full-length transcripts as a function of UTP concentration were nearly superimposable, except for a slight predominance of the full-length transcripts at the lowest UTP concentrations (Fig. 3B). Indeed, the ratio of short transcripts to full-length transcripts was nearly identical at each UTP concentration, with the exception of the lowest concentrations of UTP, where it was slightly lower (see of st/FL, top of Fig. 3A: compare lane 13 to lanes 1–3). If limiting UTP concentration contributed significantly to short transcript formation, the opposite would be expected, i.e. the ratio of short to long transcripts would be higher at the lowest concentrations of UTP. This is consistent with the observation that the short transcripts are not observed when the various IST- mutants are transcribed, which should be equally susceptible to such artifactual pausing or termination.

Wild-type Short Transcripts and Short Transcripts Carrying the ABC Mutation Are Equally Stable in Vitro—The ABC mutation changes sequences downstream of the transcription start site that are part of the short transcripts. These sequence changes maintain the predicted secondary structure of the short transcripts. Moreover, in nuclear run-on assays in vitro, which measure the rate of RNA synthesis during a time period believed to be too short to allow for significant RNA turnover, the ABC mutant does not support the accumulation of short transcripts, suggesting that the defect is caused by reduced transcription rather than by a decreased stability of the short transcripts (4). Nonetheless, the possibility remained that, in vitro, we failed to detect short transcripts derived from the
msABC/ML construct because of decreased stability. Therefore, we compared the relative stabilities of wild-type short transcripts and short transcripts carrying the ABC mutation in the in vitro transcription system. Wild-type short transcripts and short transcripts with the ABC mutation were synthesized with bacteriophage T7 RNA polymerase and incubated in reactions identical to the in vitro transcription except that the UTP was not radiolabeled. At the time points indicated, aliquots were withdrawn from the reactions and the RNAs fractionated on a sequencing gel. The bands corresponding to the short transcripts were then quantitated with a Fuji BAS1000 PhosphorImager. The counts present at time 0 were set at 100%.

IST-directed Promoter-proximal Transcription Represents a Significant Fraction of Total in Vitro Transcription—High levels of background bands around the 65-nt marker made it difficult to interpret the lower part of the gels used to resolve the in vitro transcripts. To determine whether additional IST-dependent short transcripts might be located in this region of the gel, and to map the short and long transcripts more accurately, we analyzed the products of an in vitro transcription reaction by an RNase protection assay with unlabeled antisense riboprobes (see “Experimental Procedures”). As illustrated in Fig. 5A, in a run-off assay the pHIV-1/R/ML and the msABC/ML constructs gave the expected full-length transcripts (lanes 5 and 6). As determined by quantitation of the bands with a PhosphorImager, the amounts of full-length transcripts synthesized by the msABC/ML mutant represented about 60% the amounts of full-length transcripts synthesized by the wild-type construct. A similar moderate reduction of full-length transcripts is observed with this mutant in vivo, presumably because, in addition to inactivating the DNA sequences required for activation of short transcript synthesis, the ABC mutations also compromise sequences that contribute to basal transcription (4). As expected, the pHIV-1/R/ML wild-type construct, but not the msABC/ML construct, which contains a debilitated IST, produced the short transcript doublet (lanes 5 and 6, bands labeled st). In addition, the pHIV-1/R/ML construct gave stronger signals in the 65-nt range than the msABC/ML construct, suggesting that some of these mobility bands may represent IST-dependent short transcripts.

We then analyzed the products of a similar in vitro transcription reaction by RNase protection with probes derived from the pHIV-1/ML/T3/ΔEco and pABC/ML/T3/ΔEco constructs. The pHIV-1/ML/T3/ΔEco construct as well as the location of the probes, which extend from positions −146 to +329 relative to the HIV-1 transcription start site, and the protected full-length and short transcripts, are depicted in Fig. 1B. With both templates, we observed protected RNAs of approximately 329 nt, the protected length expected for full-length transcripts (Fig. 5A, lanes 1 and 3, band labeled FL). In addition, the wild-type pHIV-1/R/ML template, but not the IST mutant msABC/ML template, gave rise to a protected doublet strikingly similar to the doublet observed in the run-off assay (compare lanes 1 and 5). Significantly, the wild-type template also gave rise to a number of bands in the 88–95- and 50–65-nt range that were not present with the IST mutant msABC/ML template (compare lanes 1 and 3). Thus, these transcripts, too, depend for their synthesis on an intact IST element, and part of the signal in the 50–65-nt range observed in the run-off assay (lane 5) actually corresponds to IST-dependent short transcripts partially obscured by background bands.

As shown in Fig. 5B, PhosphorImager quantitation of the pHIV-1/R/ML signals corresponding to the 329-nt full-length transcripts and those corresponding to transcripts shorter than 95 nt (see Fig. 5A, lane 1) revealed that the ratio of short to full-length RNA molecules is approximately 15:1. Thus, consistent with previous observations (11), there is a strong polarity of transcriptional activity from promoter-proximal to promoter-distal locations. In addition, comparison of the amounts of short transcripts obtained with the wild-type IST construct pHIV-1/R/ML and the mutated IST construct msABC/ML reveals that, after correction for the effect of the ABC mutations on general transcription and for the additional (radiolabeled) U residues present in the mutated RNAs (see Fig. 1A), the activity of IST accounts for approximately 70–80% of the promoter-proximal transcription detectable in this assay (Fig. 5B). Together, these results suggest that under these conditions IST-directed transcription represents a significant portion of the total in vitro transcription derived from the HIV-1 promoter. It has been shown previously that the HIV-1 transcriptional activator Tat increases the levels of full-length transcripts but reduces the levels of the IST-dependent short transcripts (2, 4). We therefore tested whether addition of Tat to the in vitro transcription reactions would affect the levels of short transcripts in this manner. As shown in Fig. 5A, under our transcription conditions optimized for short transcript synthesis, addition of Tat trans-activated the full-length transcripts only poorly (compare lane 2 to lane 1 and lane 4 to lane 3, band labeled FL). However, consistent with the in vivo observations, the weak bands in the 88–95-nt range, the 83–85-nt short transcript doublet, and the shorter transcripts were reduced in the presence of Tat (compare lanes 2 and 1). Thus, the in vitro
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The Short Transcripts Are Synthesized by RNA Polymerase II—Short, nonpolyadenylated RNAs are frequently synthesized by RNA polymerase III. Thus, an intriguing possibility was that the HIV-1 short transcripts were also synthesized by this RNA polymerase. To address this question, we determined the sensitivity of short and long transcript synthesis to various concentrations of the fungal toxin α-amanitin. As depicted in Fig. 6A, synthesis of both the full-length and short HIV-1 transcripts was sensitive to the low concentrations of α-amanitin that typically inhibit RNA polymerase II transcription (lanes 2–5). In contrast, the synthesis of VAI RNA, an RNA polymerase III transcript, was inhibited only at the high concentrations of α-amanitin that typically inhibit RNA polymerase III transcription (Fig. 6B). Thus, like the full-length transcripts, the HIV-1 short transcripts are synthesized by RNA polymerase II in vitro.

Synthesis of the Short but Not the Full-length Transcripts Is Resistant to DRB—The purine nucleoside analog DRB inhibits the elongation phase of RNA polymerase II transcription in vitro (12). Since IST appears to direct the formation of transcription complexes incapable of efficient transcription elongation, an intriguing possibility is that the synthesis of the short transcripts is DRB-resistant. To address this question, in vitro transcription reactions were performed with the wild-type and two IST mutant templates in the absence or presence of DRB at a concentration that has been shown to inhibit formation of HIV-1 full-length transcripts in vitro (11). As expected, DRB abolished the synthesis of the 630-nucleotide run-off transcript (Fig. 7, compare lanes 2–4 and 6–8, bands labeled FL). In contrast, it had no effect on the synthesis of the IST-dependent short transcripts (compare lanes 2–4 and 6–8, bands labeled st). This observation indicates that the synthesis of the full-

assay faithfully reproduces this important in vivo characteristic of short transcripts, further suggesting that the short transcripts detected in vitro represent IST activity. Because the properties of the 83–85-nucleotide doublet were representative of the total population of short transcripts revealed in the RNase protection assay, IST activity in vitro was henceforth assayed by accumulation of this doublet in the simpler run-off assay.

The Short Transcripts Are Synthesized by RNA Polymerase III—In the absence of DRB, synthesis of both the full-length and short HIV-1 transcripts was abolished only at the high concentrations of α-amanitin that typically inhibit RNA polymerase III transcription (lanes 2–5). In contrast, the synthesis of VAI RNA, an RNA polymerase III transcript, was inhibited only at the high concentrations of α-amanitin that typically inhibit RNA polymerase III transcription (Fig. 6B). Thus, like the full-length transcripts, the HIV-1 short transcripts are synthesized by RNA polymerase II in vitro.

Synthesis of the Short but Not the Full-length Transcripts Is Resistant to DRB—The purine nucleoside analog DRB inhibits the elongation phase of RNA polymerase II transcription in vitro (12). Since IST appears to direct the formation of transcription complexes incapable of efficient transcription elongation, an intriguing possibility is that the synthesis of the short transcripts is DRB-resistant. To address this question, in vitro transcription reactions were performed with the wild-type and two IST mutant templates in the absence or presence of DRB at a concentration that has been shown to inhibit formation of HIV-1 full-length transcripts in vitro (11). As expected, DRB abolished the synthesis of the 630-nucleotide run-off transcript (Fig. 7, compare lanes 2–4 and 6–8, bands labeled FL). In contrast, it had no effect on the synthesis of the IST-dependent short transcripts (compare lanes 2–4 and 6–8, bands labeled st). This observation indicates that the synthesis of the full-

FIG. 5. IST-dependent transcription represents a significant fraction of the total transcription from the HIV-1 promoter in vitro. A, the templates indicated above the lanes were linearized with NcoI and used to program a HeLa cell nuclear extract in the presence of radiolabeled [α-32P]UTP. The transcription reactions were identical to those described in Fig. 2, except that poly(dG-dC):poly(dG-dC) was added to a concentration of 750 ng/μl, and poly(I):poly(C) to 175 ng/μl. In lanes 2 and 4, 125 ng of Tat (recombinant protein purified from E. coli, generously provided by M. Laspi, Dartmouth School of Medicine) were added to the reactions. In lanes 1–4, the resulting RNAs were then hybridized to an excess of unlabeled riboprobe hybridizing to the –146 to +329 HIV-1 region, single-stranded RNA was digested with RNase T1, and the protected RNA fragments were resolved on a sequencing gel. In lanes 5 and 6, the radiolabeled RNAs produced in vitro were directly fractionated on the same sequencing gel. In lanes 5 and 6, the bands labeled FL have the expected length for full-length run-off RNAs initiated at the HIV-1 promoter. In lanes 1–4, the bands labeled FL have the expected length for transcripts protected from position +1 to position +329, where the complementarity to the antisense riboprobe ends. The bands corresponding to short transcripts. The sizes of the DNA markers are indicated on the right. B, quantitative analysis of promoter-proximal and full-length transcription detected in A. The bands corresponding to full-length transcripts or to transcripts shorter than 95 nt in lanes 1 and 3 of panel A were quantitated with a Fuji BAS1000 PhosphorImager. The numbers were normalized with respect to the number of U residues present in each type of RNA molecules. In addition, we corrected the short transcript signal obtained for lane 3 for the negative effect of the ABC mutations on general transcription by multiplying it by the ratio of full-length transcripts observed with the wild-type construct (lane 1) versus full-length transcripts obtained with the IST mutant construct (lane 3). The amount of transcripts shorter than 95 nt obtained with the wild-type IST construct (lane 1 of A) was then set at 100%. The relative amount of full-length transcripts (329 nt in length) obtained with the same construct is shown, as well as the approximate contribution of the activity of IST to promoter-proximal transcription (cross-hatched portion of the bar).
length transcripts is not a prerequisite for the synthesis of the short transcripts, and thus confirms that the short transcripts do not arise from degradation or processing of long transcripts. In addition, the amounts of short transcripts were not increased in the presence of DRB, and no short transcripts were induced when IST− mutants msABC/ML and IST-ML were transcribed in the presence of DRB (compare lanes 7 and 8 to lane 6; see also Fig. 9B, lanes 3 and 7). Therefore, DRB does not appear to convert elongation competent transcription complexes into elongation incompetent ones. It rather seems to inhibit the assembly of elongation competent transcription complexes at the promoter. And most importantly, this observation supports a model in which short and full-length transcripts are the products of two types of transcription complexes with distinct biochemical properties: unlike formation of the transcription complex giving rise to full-length transcripts, formation of the transcription complex giving rise to the short transcripts does not require the activity of factor(s) inhibited by DRB.

Differential Reaction Requirements for Full-length and Short Transcript Synthesis—While optimizing conditions for the in vitro transcription reactions, we noticed that various reaction parameters affected the synthesis of short and long transcripts differentially. For example, Fig. 8, A and B, show that increasing amounts of either poly(I)poly(C) or poly(dG-dC)poly(dG-dC) inhibited full-length transcription but stimulated the synthesis of the short transcripts over a broad concentration range. Similarly, synthesis of short transcripts had a much sharper MgCl2 optimum than that of the full-length transcripts (Fig. 8C, lanes 3–10, compare FL and st bands). No short transcripts were induced by varying the concentrations of the above nucleic acid competitors or MgCl2 when the mutant IST construct msABC/ML was transcribed (data not shown).

Fig. 9A shows the effects of adding increasing amounts of NaCl to the transcription reaction. When the salt concentration in the reaction was raised by adding small increments of NaCl in addition to the 50 mM KCl contributed to the reactions by the nuclear extract, synthesis of the full-length transcripts decreased steadily (lanes 2–7). In contrast, the synthesis of short transcripts was first stimulated to a maximum at 80 mM salt (30 mM NaCl added, lane 4) and was inhibited only at higher salt concentrations (lanes 5–7). To rule out the possibility that the stimulatory effect of increased NaCl on short transcript synthesis was due to a conversion of full-length transcripts into short transcripts, for example, by inhibition of transcript elongation or induction of an RNA processing activity, we wished to test whether addition of NaCl to the reaction would modulate short transcript synthesis in the absence of the full-length transcripts. In vitro transcriptions were performed in the presence of different salt concentrations and the nucleoside analog DRB, which, as shown above (Fig. 7), selectively inhibits formation of the full-length transcripts. As expected, in the absence of DRB, raising the total salt concentration to 80 mM by adding 30 mM NaCl to the reaction severely reduced the level of the full-length transcripts and, conversely, stimulated the level of the short transcripts (Fig. 9B, compare lanes 1 and 2). Significantly, addition of NaCl did not lead to the appearance of short transcripts when the IST− template msABC/ML was transcribed in an identical experiment (compare lanes 5 and 6, and 7 and 8). Thus, the increase in short transcripts observed at increased NaCl concentrations, and probably also that observed at certain MgCl2 and nucleic acid competitor concentrations, most likely results from a stimulation of IST activity and not from a general inhibitory effect on transcription elongation or modulation of an RNA processing activity.
Together, these results demonstrate that in vitro, as in vivo (3, 4), the short and the full-length transcripts arise from separable processes that can be modulated independently. They lend further support to the notion that short and full-length transcripts are synthesized by different transcription complexes with distinct biochemical properties.

**DISCUSSION**

**The Short HIV-1 Transcripts Observed in Vitro Result from IST-dependent RNA Polymerase II Transcription**—Several lines of evidence suggest that the short transcripts observed in the in vitro transcription system reflect the activity of the IST element that we have previously characterized in vivo. First, mutations that debilitate the IST element in vivo severely reduce the accumulation of short transcripts in vitro. In the case of the msABC mutation, we have shown that this is not due to decreased stability of short transcripts carrying the ABC mutation, as these transcripts are degraded at the same rate as wild-type short transcripts. Second, as in vivo, the Tat protein decreases the level of short transcripts. Third, since the ratios

**FIG. 8.** Varying several transcription reaction parameters affects synthesis of short and full-length transcripts differentially. A, the pHIV-1/R/ML template was linearized with Ncol and used to program a HeLa cell nuclear extract. The reactions contained the indicated amounts of poly(I)-poly(C). The bands corresponding to full-length and short transcripts were quantitated with a Fuji BAS1000 PhosphorImager, and the counts present with no added poly(I)-poly(C) were set at 100%. B, the reactions were performed and analyzed as in A except that the reactions contained the indicated amounts of poly(dG-dC)-poly(dG-dC) instead of poly(I)-poly(C). C, the transcription was performed with the templates indicated above the lanes, and the MgCl₂ concentration was varied as follows: lanes 1–3 contained 2.5 mM MgCl₂ derived from the nuclear extract. In lanes 4–10, additional MgCl₂ was provided to the following final concentrations: 3.75 mM (lane 4), 5.0 mM (lane 5), 6.25 mM (lane 6), 7.5 mM (lane 7), 8.75 mM (lane 8), 10.0 mM (lane 9), 11.25 mM (lane 10). Only the top and bottom portions of the gel, which contain the bands of interest, are shown.

Together, these results demonstrate that in vitro, as in vivo (3, 4), the short and the full-length transcripts arise from separable processes that can be modulated independently. They lend further support to the notion that short and full-length transcripts are synthesized by different transcription complexes with distinct biochemical properties.
of short to full-length transcripts are similar throughout a broad UTP concentration spectrum, the short transcripts do not arise from artifactual polymerase pausing or termination due to the low UTP concentration in our assay. Finally, by selectively abolishing synthesis of the full-length transcripts but not the short transcripts with DRB or NaCl, we have also shown that the short transcripts are not breakdown products of the full-length transcripts.

There is, however, a discrepancy. Whereas in vivo, the RNase T1 protection assay reveals short transcripts protected over 58, 62, and 65 nt, RNase T1 protection of the in vitro transcripts reveals a series of molecules in the 50–65-nt range and a doublet of about 83–85 nt. These differences are likely to reflect, at least in part, differences in stability of short transcripts of different lengths in vitro and in vivo. Perhaps the very short RNAs detected in vitro are not stable in vivo because they are too short to fold into the TAR stem-and-loop structure and are thus degraded. Similarly, the longer RNAs detected in vitro may not be stable in vivo because they are processed to the base of the stem-and-loop structure. Indeed, pulse-chase studies with isolated nuclei have shown that heterogeneous, longer precursors to HIV-1 short transcripts exist in vivo, which are then shortened by a RNA processing activity (13). Thus, although the sizes of the short transcripts detected in vivo and in vitro differ, we have established a cell-free transcription assay that faithfully reproduces the transcriptional activity of the HIV-1 IST element, and it will now be possible to study the biochemical events underlying its function.

Several RNA polymerase II promoters can also direct RNA polymerase III transcription under certain circumstances. For instance, the c-Myc promoter can support the synthesis of short, RNA polymerase III transcripts both when injected into Xenopus oocytes (14), and in vitro (15). The human T-lymphotropic virus type 1 promoter can direct the synthesis of RNAs that are initiated at the same nucleotide as the RNA polymerase II transcripts but are synthesized by RNA polymerase III, apparently recruited to the promoter in part by RNA polymerase II transcription factors (16). Because the HIV-1 short transcripts are stable in vivo, it has been difficult to determine their sensitivity to α-amanitin in transfection experiments. Here, we show that, as determined by their sensitivity to low concentrations of α-amanitin, the IST-directed HIV-1 short transcripts synthesized in vitro are the products of RNA polymerase II. This suggests that in vivo, too, IST-directed transcription is mediated by RNA polymerase II.

The formation of short HIV-1 and HIV-2 transcripts in vitro was first reported by Toohey and Jones (17). In their system, efficient formation of short transcripts required the addition of Sarkosyl to the transcription assay. However, the observations that Sarkosyl induced a processing activity in the nuclear extract, and that the short transcripts appeared after the full-length transcripts, suggested that the short transcripts detected in that system resulted from processing of the full-length transcripts (17). It seems unlikely, therefore, that the short transcripts observed in that system reflected activity of the IST element. In contrast, in their in vitro studies of Tat transactivation, Laspia et al. (7) detected short transcripts of 80–85 nucleotides in length whose synthesis decreased upon the addition of Tat to the reaction. Even though we did not observe the same response of short transcript synthesis to some reaction parameters described by Laspia et al. (7) (e.g., stimulation by template preincubation, presynthesis in the absence of radiolabeled nucleotides, or addition of Sarkosyl, data not shown), it is possible that their short transcripts are related to the 83–85-nucleotide doublet detected in our assay. It also seems likely that the steep gradient of transcription downstream of the HIV-1 promoter observed previously in vitro (11) reflects in large part the activity of the IST element. Indeed, in these experiments, the addition of DRB inhibited Tat-activated transcription but had little effect on promoter-proximal transcription.

Two Types of Transcription Complexes Are Assembled at the HIV-1 Promoter—Our in vivo studies of the IST element have suggested that the HIV-1 promoter directs the assembly of two
types of transcription complexes that differ in their elongation properties (2, 4): one type, whose formation is stimulated by Tat binding to the TAR element, is elongation competent, while the second type, whose formation is stimulated by IST, is incapable of efficient elongation. Although these two types of transcription complexes do not appear to differ in their use of the TATA-box binding protein (5), they probably contain different factors. Thus, the IST element, which is not required for synthesis of the full-length transcripts, is likely to correspond to the binding site for a factor that specifically stimulates the assembly of such elongation-incompetent complexes. Indeed, the second type, whose formation is stimulated by IST, is capable of efficient elongation. Although these two types of transcription complexes do not appear to differ in their use of Tat binding to the TAR element, is elongation competent, while the second type, whose formation is stimulated by IST, is incapable of efficient elongation. Therefore, resistant to DRB. In these IST-directed transcription complexes, the RNA polymerase II carboxyl-terminal domain (21, 26), is consistent with a model in which the IST-directed transcription complexes do not depend on certain carboxyl-terminal domain kinases and are, therefore, resistant to DRB. In these IST-directed transcription complexes, the RNA polymerase II carboxyl-terminal domain would remain largely unphosphorylated, resulting in poor elongation properties. Alternatively, or in addition, the IST element may serve as an entry point for a transcription complex prone to premature transcription termination.

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