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Effects of heterologous downstream sequences on the activity of the HIV-1 promoter and its response to Tat

Michael E. Greenberg[1] and Michael B. Mathews[1,2,*]

[1]Cold Spring Harbor Laboratory, PO Box 100, Cold Spring Harbor, NY 11724, USA and [2]Department of Biochemistry and Molecular Biology, New Jersey Medical School, UMDNJ, Newark, NJ 07103, USA

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ABSTRACT

In HIV-1 infection, Tat acts at least in part to control transcriptional elongation by overcoming premature transcriptional termination. In some other genes this process is governed by DNA elements called attenuators in concert with cellular transcription factors. To understand the action of Tat more fully and explore its role as an anti-attenuator, we examined the ability of several natural and synthetic attenuation sequences to modulate transcription initiated at the HIV LTR. Fragments containing these signals were inserted downstream of the TAR element in an HIV-CAT chimera and their effects on transcription were assessed both *in vitro* and *in vivo*. Runoff transcription assays in HeLa cell extracts demonstrated that the attenuators give rise to premature termination of transcripts initiated from the heterologous HIV-LTR promoter *in vitro*. When transiently expressed following transfection into Cos cells, however, premature transcript termination at the attenuation site was not observed. Nevertheless, many of the inserted sequences exerted marked effects on CAT gene expression and on transactivation by Tat at both the RNA and protein levels. The nature and magnitude of the effects depended upon the identity of the attenuator and its orientation but only one of 16 sequences tested met the criteria for a Tat-suppressible attenuator *in vivo*. One other sequence, in contrast, severely reduced Tat-activated transcription without inhibiting basal transcription These results indicate that sequences downstream of the HIV LTR can influence its function as a promoter and its response to Tat transactivation, but lend little support to their role as attenuators *in vivo*.

INTRODUCTION

Intense efforts to control the AIDS epidemic have sparked a detailed examination of its etiological agent, the human immunodeficiency virus, HIV-1. This has led to the recognition that HIV encodes novel regulatory proteins which profoundly affect the course of viral gene expression in infected cells. One of these, an essential protein known as Tat, is a potent activator of viral transcription from the viral promoter, located in the long terminal repeat (LTR) (1–3). Early in infection, the basal level of transcription initiating at the HIV LTR is low. The binding of Tat to a structured RNA element termed TAR (the transactivation response element), located between nucleotides +15 and +45 relative to the cap site at +1, brings about a dramatic increase in viral transcription from the LTR. It has been suggested that TAR acts as an RNA enhancer element by recruiting Tat in concert with cellular protein cofactors (3–5). The precise mechanisms remain unclear, but evidence exists suggesting that Tat acts by increasing the rate of transcriptional initiation, by stabilizing transcriptional elongation, or through a combination of the two (6–13). Since the LTR is disposed to produce abortive short transcripts (6,14–16), the hypothesis has been advanced that Tat might function to overcome the action of an attenuator element (17).

Premature transcriptional termination (or attenuation) is increasingly recognized as a means of gene regulation with far-reaching consequences in eukaryotes (18), as well as prokaryotes (19). Attenuation contributes to the regulation of several eukaryotic proto-oncogenes and viral genes (for reviews, see 20–23). Nucleotide sequences have been defined which act as specific attenuators able to cause pausing and premature termination when placed in the path of the transcription complex (20,22). Some of these sequences have been characterized with regard to their predicted RNA secondary structures and the specific elongation factors required to promote efficient readthrough by the polymerase complex (20,24–27). For example, transcripts from sequences conferring an elongation block from several viruses are predicted to form stable stem–loop structures followed by a run of uridine residues. Both motifs have been shown to contribute to polymerase stalling, while certain oncogene transcripts contain a uridylate-rich tract with no apparent secondary structure (20,24,27,28). The conditional transcription termination imposed by these blocks can be overcome by modifying the arrested RNA polymerase complex to an anti-terminating form, in some cases by recruiting cellular elongation factors. Several cellular factors play an essential role in stabilizing the elongation complex; these include transcription factors TFIIF and TFIIS, elongin or SIII, and ELL (29–31). That uncontrolled transcriptional elongation may contribute to certain forms of human cancer is suggested by the recent finding that elongin is negatively regulated by the product of the tumor suppressor gene VHL (32).

Tat increases elongation efficiency both *in vivo* and in a cell free transcription system (6–8,10,11). In the absence of Tat, there is an accumulation *in vivo* and *in vitro* of short transcripts

*To whom correspondence should be addressed at: Department of Biochemistry and Molecular Biology, New Jersey Medical School, UMDNJ, 185 South Orange Avenue, Newark, NJ 07103, USA. Tel: +1 973 972 4411; Fax: +1 973 972 5594; Email: mathews@umdnj.edu

terminating at the 3′ border of the TAR element (6,15,33). TAR adopts a stem–loop conformation (34) which resembles some attenuators structurally. Furthermore, it can function as an attenuator *in vitro*, causing polymerase pausing and termination (35). By analogy with the bacteriophage λ anti-terminator N, which also binds a promoter-proximal RNA target via an arginine-rich motif (17,36,37), it was postulated that Tat acts by recruiting specific host factors which modify the transcription complexes to a more processive form (38). Evidence consistent with this view has been drawn from several studies, of which two in particular reported attenuator readthrough. First, premature termination was elicited *in vitro* by insertion of a synthetic attenuator, specifying a stable RNA stem–loop structure followed by a run of Us, between the HIV LTR and downstream viral sequences. The addition of purified Tat protein increased transcription through the attenuator (12), suggesting that Tat allows complexes to read through heterologous transcription blocks. Second, Tat was shown to allow readthrough of a natural c-myc attenuator in cells transfected with a plasmid containing the TAR element inserted upstream of the promoter-proximal attenuation site in the c-myc gene (39).

As an alternative approach toward defining the activity of Tat, we examined its capacity to function as an anti-terminator when heterologous DNA sequences encoding naturally-occurring attenuation signals were placed downstream of the TAR element in the HIV LTR. Since attenuators differ in their structure, properties and interactions with protein factors, implying that they operate by differing mechanisms (24,26), Tat might be able to overcome some attenuators but not others, permitting inferences to be drawn about the mechanism of Tat's action. We therefore isolated several DNA fragments encoding attenuators that had been characterized in cell-free systems, inserted them individually in both orientations into a plasmid vector between the TAR element and the CAT reporter gene, and assayed for premature transcription termination *in vivo* and *in vitro*. Although the attenuators functioned *in vitro* when placed downstream of the HIV-1 promoter, technical limitations precluded the simultaneous observation of both the Tat response and attenuation in the cell-free reaction. Transfection experiments in Cos cells showed that several of the inserts affect HIV transcription (either basal or Tat-activated, or both), but suppression of attenuation *in vivo* was not generally observed.

MATERIALS AND METHODS

Construction of plasmids

LTR-CAT constructs were derived from a previously described plasmid, pH2 (15). Minimal DNA sequences reported to confer attenuation *in vitro* were excised from the viral and cellular genes listed in Table 1 and *Hin*dIII linkers were attached. To construct the SV40 attenuator-containing plasmid, the SV40 containing vector pSV010 (40) was subjected to 10 cycles of PCR using flanking primers, d(CACAAGCTTTCAGGCCATGGTGC) and d(CACAAGCTTGTCAACAGTATCTTCCC), which span the attenuation sequence with *Hin*dIII sites (underlined) added near their termini for cloning purposes. The *Hin*dIII fragments were ligated into the unique *Hin*dIII site at position +79 of pH2. Plasmids carrying inserts in both sense and antisense orientations were characterized by sequence analysis. Plasmids containing the attenuator encoding inserts are named according to the gene from which the insert was subcloned, with the suffix '/r' designating reverse orientation. For synthesis of RNA probes, segments from the pH2 derivatives were subcloned into pGEM-1 or pGEM-3 vectors (Stratagene). The HIV LTR–attenuator–CAT sequence, from the *Sca*I site at –135 to the *Eco*RI site at +329, was inserted into the *Eco*RI–*Sma*I window of the pGEM polylinker region (Fig. 1). pGEM PCNA CAT (41) was used to synthesize an RNA probe for protecting downstream CAT sequences.

Transfection assays

Calcium phosphate transfections were performed as described previously (9). Transfection mixtures contained 3 μg of attenuator plasmid and 0.5 μg of a control plasmid pαA+C (42). Tat was generated by inclusion of 1.0 μg of pCMV-Tat in the transfection mix. The DNA was made up to 20 μg with herring sperm DNA and overlayed onto 80% confluent monolayers of Cos cells in 60 mm tissue culture dishes. At 16 h post-transfection, cells were washed twice with fresh medium then overlayed with 5 ml of medium. Cells were incubated at 37°C.

Analysis of cellular RNA

Total RNA was harvested at 48 h post-transfection by the RNAzol method (43). Briefly, cells were washed twice on ice with PBS and scraped into 0.5 ml RNAzol made up of water-equilibrated phenol (USB ultrapure), guanidinium thiocyanate and sarcosyl. RNA was subsequently precipitated in isopropanol, redissolved in water, extracted repeatedly with phenol:chloroform (1:1) and reprecipitated. RNA concentration was determined by A_{260} measurement. Antisense RNA probes were synthesized with T7 RNA polymerase and [α-^{32}P]UTP from pGEM vectors linearized with *Sal*I, and purified on sequencing gels. To perform the RNase protection assay, 5 μg RNA was hybridized overnight with 5×10^5 c.p.m. of RNA probe at 40°C in 30 μl 80% formamide, 40 mM PIPES, pH 6.7, 400 mM NaCl and 1 mM EDTA. Single-stranded RNA was then digested with 10 μg/ml RNase T1 in 300 μl 10 mM Tris, pH 6.7, 400 mM NaCl and 1 mM EDTA for 1 h at room temperature, SDS and proteinase K were added to 0.5% and 167 μg/ml, respectively, and incubation was continued for 15 min at 37°C. RNA was extracted with phenol:chloroform, precipitated with ethanol, and analyzed by electrophoresis in 8% polyacrylamide–7 M urea sequencing gel. Protected RNA fragments were detected by autoradiography using an intensifying screen. Quantitation was performed with a Fuji phosphorimager.

In vitro transcription

Transcription was performed in HeLa cell nuclear extract according to previously described methods (24). Briefly, 10 μl of nuclear extract (80–100 μg protein), linearized DNA (0.125 μg) and 4 mM creatine phosphate were incubated for 30 min at 30°C in a final volume of 15 μl. Following pre-initiation, ATP, CTP and GTP were added to a final concentration of 62.5 μM each together with 20 μCi of [α-^{32}P]UTP in a volume of 5 μl and pulse labeling was conducted for 1.5 min. For the elongation step, 2 μl of each of the four rNTPs (final concentration 0.6 mM) were added and the reaction was allowed to proceed for an additional 30 min at 30°C. Reactions were stopped by addition of 100 μl of 20 mM EDTA, 0.2% SDS, and 200 μg/ml proteinase K and 30 μg tRNA. After incubation at 65°C for 15 min, RNA was extracted with phenol:chloroform and with chloroform, and then was precipitated with ethanol. Transcription products were analyzed in 6% polyacrylamide–7 M urea gels.

Table 1. Attenuator elements

Attenuator insert	Gene location[a]	Expected attenuated RNA (nt)[b]	Structure/ motif	Reference
MVM	48–194	182	stem–loop, poly U	55
SV40	329–476	173	stem–loop, poly U	47
AdML	6094–6241	230	stem–loop, poly U	56
c-fos	834–1011	198	stem–loop, poly U	27
c-myc	1978–2160	179	poly U	28
ODC-1	161–343	139	poly U	45
ODC-2	1486–1687	180	poly U	45
synthetic	–	129	stem–loop, poly U	12

[a]Nucleotide number according to numeration in cited reference.
[b]Length in nucleotides between the HIV start site and the attenuation site published in the literature.

RESULTS

It has been suggested that Tat converts RNA polymerase II transcription complexes initiating at the HIV promoter into a more processive form (3), possibly through recruitment of novel host factors. Consistent with such a mode of action, Tat has been shown to augment readthrough of attenuation signals in two cases (12,23). Several types of attenuation signal have been identified in cellular and viral genes through cell-free transcription studies. Some of these attenuator sequences caused premature transcriptional termination *in vitro* when placed under the control of a heterologous promoter such as the adenovirus major late (AdML) promoter (24,27,44). We have examined the ability of such sequences, from viral and cellular sources, to attenuate transcription from the HIV LTR *in vivo*, and have tested their influence on transactivation by Tat.

Experimental design

We isolated several attenuators from three cellular genes, c-myc (myc), c-fos (fos) and ornithine decarboxylase (ODC), and three viral genomes, adenovirus major late transcription unit (AdML), simian virus 40 (SV40) and minute virus of mice (MVM). In addition, we utilized a synthetic attenuator sequence (GC). The properties of these sequences had all been characterized previously by *in vitro* transcription experiments (12,27,28,45–48). The attenuators were individually subcloned into pH2, a plasmid containing the HIV-1 LTR driving the CAT reporter gene, together with the SV40 origin of replication. The inserts were positioned downstream of the HIV-1 TAR element, between the LTR and the CAT reporter gene (Fig. 1A). For ease of reference, the pH2 containing attenuator chimeras are referred to by the name of the gene from which the subcloned fragment was isolated. Inserts were tested in both orientations, the suffix '/r' denoting the sense opposite to that of its natural gene. Thus, myc and myc/r refer the attenuator sequence isolated from the human c-myc gene cloned in sense and antisense orientation into pH2. Table 1 summarizes pertinent information about the attenuator elements.

Attenuation leads to a reduction in downstream transcription and, in some cases, to the accumulation of promoter proximal transcripts. We first verified the ability of selected inserts to effect transcriptional pausing *in vitro* when situated downstream of the heterologous HIV LTR promoter. Cell-free transcription assays were conducted with a subset of the constructs in the presence of 0.4 M KCl, a condition that favors attenuation. Sequences from the myc, fos, ODC-2 and AdML genes all gave rise to both

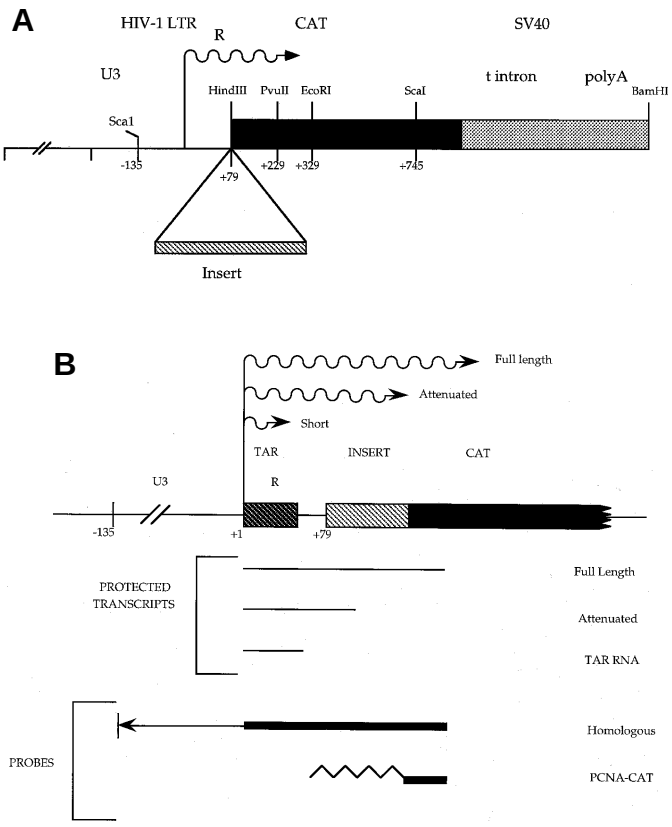

Figure 1. Schematic of constructs, transcripts and probes. (**A**) The pH2 parent plasmid was used to construct the attenuator constructs (15). Minimal attenuator-containing fragments were isolated from sources listed in Table 1, *Hin*dIII linkers attached, and ligated in both orientations into the unique *Hin*dIII site at position +79 between the LTR promoter and CAT reporter gene. (**B**) RNA transcripts synthesized from the pH2-attenuator plasmids were detected using riboprobes. Two species of RNA were known to be produced from these plasmids, full length transcript and short RNA terminating in the vicinity of TAR. The presence of a third species, terminating within the attenuator insert, was monitored using homologous riboprobes capable of protecting RNA corresponding to the cloned inserts plus flanking sequences within the pH2 vector. The PCNA-CAT riboprobe (41) was used in RNase protection experiments to protect CAT RNA sequences downstream of the attenuator inserts, yielding a protected band of 150 nt.

full-length runoff transcripts and shorter transcripts of the sizes expected for attenuated RNA fragments (data not shown). Attempts to assess the efficiency with which Tat-activated transcription reads through these attenuators *in vitro* failed, however, ostensibly because the relatively high salt concentration necessary to bring about attenuation *in vitro* is incompatible with transactivation by Tat. Thus, Tat stimulated transcription in the presence of 0.02 or 0.2 M KCl but not at 0.4 M, whereas attenuation was observed only in the latter condition (data not shown). In contrast, we were able to reproduce the results reported by Graeble *et al*. (12) demonstrating Tat-activated transcription together with attenuation at a synthetic attenuator *in vitro* at moderate concentrations of KCl. This difference in behavior probably reflects the greater strength of the synthetic attenuator relative to the natural attenuators, allowing the former to function *in vitro* under milder conditions (24).

Effects of attenuator sequences and Tat on readthrough *in vivo*

To examine the effects of the attenuator sequences *in vivo*, we introduced the chimeric genes into Cos cells by transfection, in the presence or absence of a Tat expression vector, pCMV-Tat (50). Transient expression assays were conducted in duplicate with the entire repertoire of attenuators (Figs 2–4). Because CAT mRNA translation is influenced by the inserted upstream sequences (data not shown), we used RNase protection assays rather than CAT enzyme assays to monitor transcript accumulation. Total cellular RNA was harvested and examined for readthrough transcription using a downstream probe (PCNA-CAT) complementary to the 5′ terminal 250 nt of the CAT transcript (Fig. 1B). As an internal control, another plasmid, pαA+C (encoding α-globin), was included in all of the transfections (42). Relative RNA levels were quantified from several reiterations of the experiments, and are summarized in Figure 5.

Representative results for the GC and the AdML inserts are shown in Figure 2. The GC attenuator is identical to the synthetic sequence which had been shown to bring about polymerase pausing *in vitro* (12) but had not previously been tested *in vivo*. It is expected to give rise to an RNA element that forms a stable stem–loop structure followed by a run of nine uridine residues (12). RNase protection assays (Fig. 2A), revealed no significant difference in the level of CAT gene readthrough RNA between the GC plasmid (lanes 2) and the parental reporter plasmid pH2 (lanes 1), suggesting that the synthetic terminator did not cause transcriptional attenuation in this assay. The RNA levels were similarly unchanged when the insert was present in the reverse orientation (GC/r; lanes 3). Tat transactivated expression from the parental plasmid by 12–15-fold (compare lanes 4 with lanes 1). Likewise, Tat increased the levels of CAT RNA expression from the GC and GC/r plasmids to a similar degree (compare lanes 5 with 2). Thus, this insert had no discernible influence on either the level of downstream transcription or the response to Tat in transient expression assays.

The GC sequence is an artificial attenuator designed to represent a class of signals consisting of an RNA hairpin followed by an oligouridylate run (Table 1), which are effective in causing premature termination in runoff reactions *in vitro* (24). Although the GC sequence functioned as an efficient attenuator *in vitro*, it was possible that it lacks features needed for such function *in vivo*. We

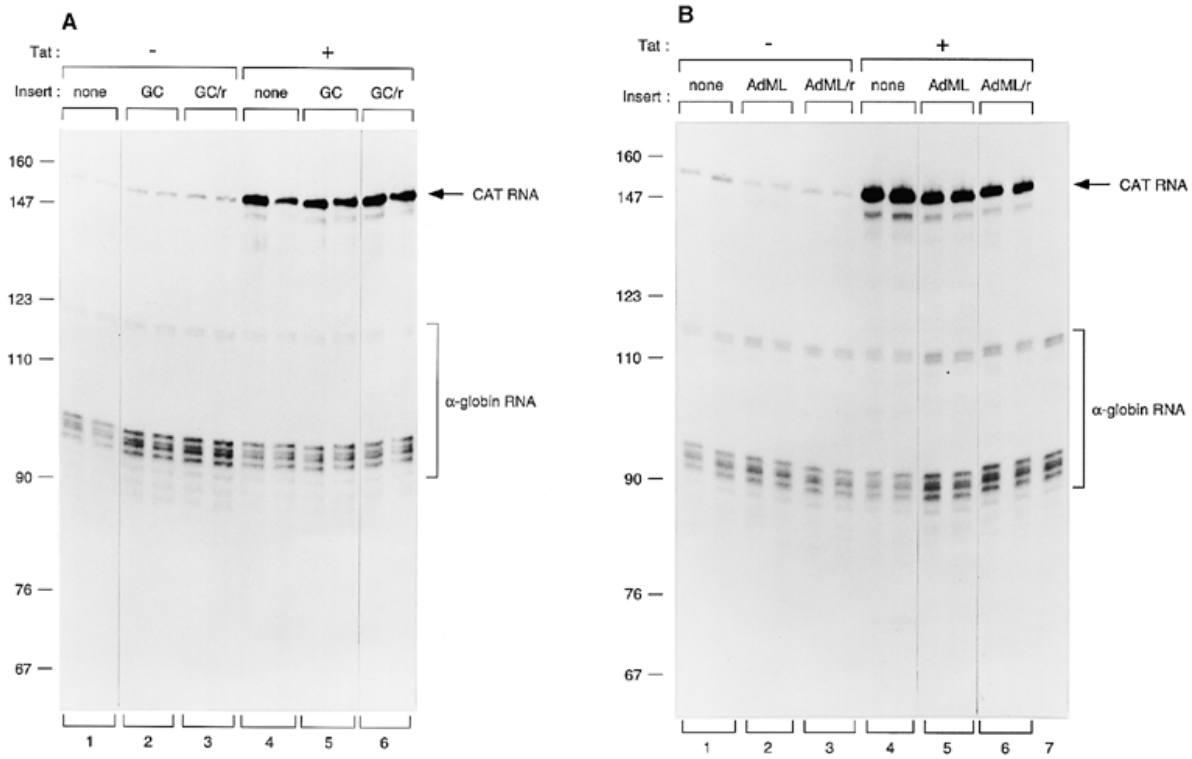

Figure 2. Transcription of plasmids containing the GC (synthetic) and AdML attenuators. (**A**) pH2 plasmid (lane 1), GC insert (lane 2) and reverse GC (GC/r) were transfected into Cos cells alone (lanes 1–3) or together with CMV-Tat (lanes 4–6). RNA harvested at 48 h post-transfection was hybridized to the PCNA-CAT riboprobe. The products were subjected to RNase T1 digestion and separated in a 6% polyacrylamide–7 M urea gel. The protected CAT sequence product and α-globin control products are marked. (**B**) Similar RNase protection analysis of RNA from cells transfected with pH2, AdML and AdML/r alone (lanes 1–3), or together with CMV-Tat (lanes 4–6) RNA from mock transfected cells was analyzed in lane 7.

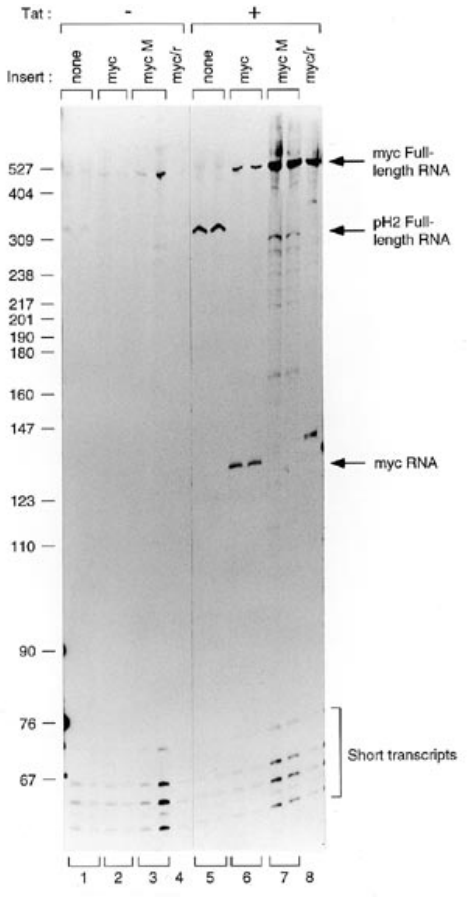

Figure 3. Effects of myc sequence on transcripts from the HIV LTR. RNA isolated from transfected COS cells was analyzed by RNase protection using homologous riboprobes. Transfections contained pCMV Tat as indicated, together with pH2 (lanes 1 and 5), myc (lanes 2 and 6), mycM (lanes 3 and 7) or myc/r (lanes 4 and 8). The myc RNA product marked corresponds to a spliced transcript.

therefore applied the same assay to the natural AdML sequence. This sequence gives rise to short RNA transcripts prematurely terminated at a run of four U residues preceded by an imperfect stem–loop structure (20,49). Compared to pH2 (Fig. 2B, lanes 1), this insert gave a 2-fold reduction in CAT RNA expression in both the sense (AdML, lanes 2) or the reverse (AdML/r, lanes 3) orientation. Tat transactivated all three plasmids to approximately equal extents (lanes 4–6). The relative levels of RNA transcribed through the AdML attenuator remained about half the level produced by the pH2 plasmid. These findings indicate that the AdML sequence exerts an inhibitory effect on downstream RNA levels and that, although Tat promotes transcription through these sequences, it does not restore transcription to the level observed when no insert is present. The orientation-independence of the inhibition may signify that it is due to a DNA element and not to the attenuator sequence itself which is thought to function as an RNA element (20).

Two other viral attenuators, from SV40 and MVM, are of the stem–loop-oligo U type. They also failed to reduce expression of CAT RNA, although in the sense orientation the SV40 sequence interfered with Tat transactivation (Fig. 5).

The myc attenuator signal differs from these sequences in its structure and properties (Table 1). When tested in a similar way, the myc insert had little effect on CAT mRNA accumulation in the absence of Tat, but reduced Tat transactivation to <5-fold. In the reverse orientation, myc/r exerted a slight inhibitory effect in both the presence and absence of Tat (Fig. 5). Despite the suggestion of a specific action on both basal and Tat-activated transcription complexes, further analysis pointed to a post-transcriptional explanation.

In *Xenopus* oocytes, the attenuation site has been mapped to an oligo U stretch near the exon 1/intron 1 of the c-myc transcript (28). To determine whether attenuation occurred at this site in our system, we conducted RNase protection assays using a set of homologous probes complementary to the 5′ end of each transcript including the insert and extending into the CAT coding sequences (Fig. 1B). RNA from cells transfected with pH2 gave rise to a protected fragment of 329 nt, as expected, and quantitative comparison gave evidence of the Tat transactivation effect (Fig. 3, lanes 1 and 5). Transfection with the plasmid containing the myc insert yielded a protected fragment of 560 nt, longer than the fragment derived from pH2 by the length of the c-myc attenuation insert (lanes 2 and 6). A fragment of similar size was also produced when the myc insert was present in the reverse orientation (myc/r; lanes 4 and 8). With the myc insert in the sense orientation, an additional protected fragment, of ~130 nt, was generated. This smaller fragment was not observed when the insert was in the reverse orientation. Assuming that the 5′ end of the smaller RNA fragment is located at the nt +1 site of the HIV LTR promoter, its 3′ end would map close to the intron 1/exon 1 border, of the myc insert rather than at the attenuation site 23 nt downstream. This raised the possibility that the 130 nt protected fragment results from an alternative splicing event, utilizing the 5′ splice donor site CAG/GT contained within the c-myc attenuation sequence, possibly together with an acceptor in the SV40 t intron (Fig. 1A), leading to the deletion of intervening CAT and plasmid sequences. To test this interpretation, the splice site was changed to CAG/CT, which would be expected to inactivate it. The mutation indeed eliminated the appearance of the short protected fragment, leading to an increase in full-length protected RNA (mycM; lanes 3 and 7). It also restored readthrough transcription to levels comparable with pH2 (Fig. 5). We conclude that the reduction in CAT RNA expression caused by the c-myc insert is due to splicing instead of attenuation.

Similar observations were made with the fos insert, which spans the c-fos intron 1/exon 1 border (Fig. 5). A fragment of 110 nt was protected, mapping the 3′ end of the short RNA precisely to the intron 1/exon 1 border. This transcript also disappeared when the GT splice donor site was mutated to CT (data not shown) with a concomitant increase in the level of readthrough transcript. Neither the myc nor fos inserts gave any indication of acting as attenuators in this transfection assay.

The ornithine decarboxylase gene contains two attenuator elements, ODC1 and ODC2, one of which (ODC2 in the sense orientation) gave a strong attenuation effect *in vitro* (data not shown). Surprisingly, the strongest effect *in vivo* was elicited by this element inserted in the reverse orientation (ODC2/r; Fig. 4A). When tested *in vivo*, the ODC1 insert increased readthrough transcription regardless of its orientation and of the presence or absence of Tat (Fig. 5). The ODC2 insert had little effect in the sense orientation, but in the reverse orientation it reduced readthrough transcription ~3-fold (Fig. 4A, lanes 1–3). Cotransfection of ODC2/r with pCMV-Tat gave an exceptionally great stimulation, restoring the RNA to a level similar to that obtained with pH2 and ODC2 (lanes 4–6). Such behavior was consistent with that expected if the ODC2/r sequence contained an attenuator that Tat can override. These observations were confirmed through RNase protection assays using

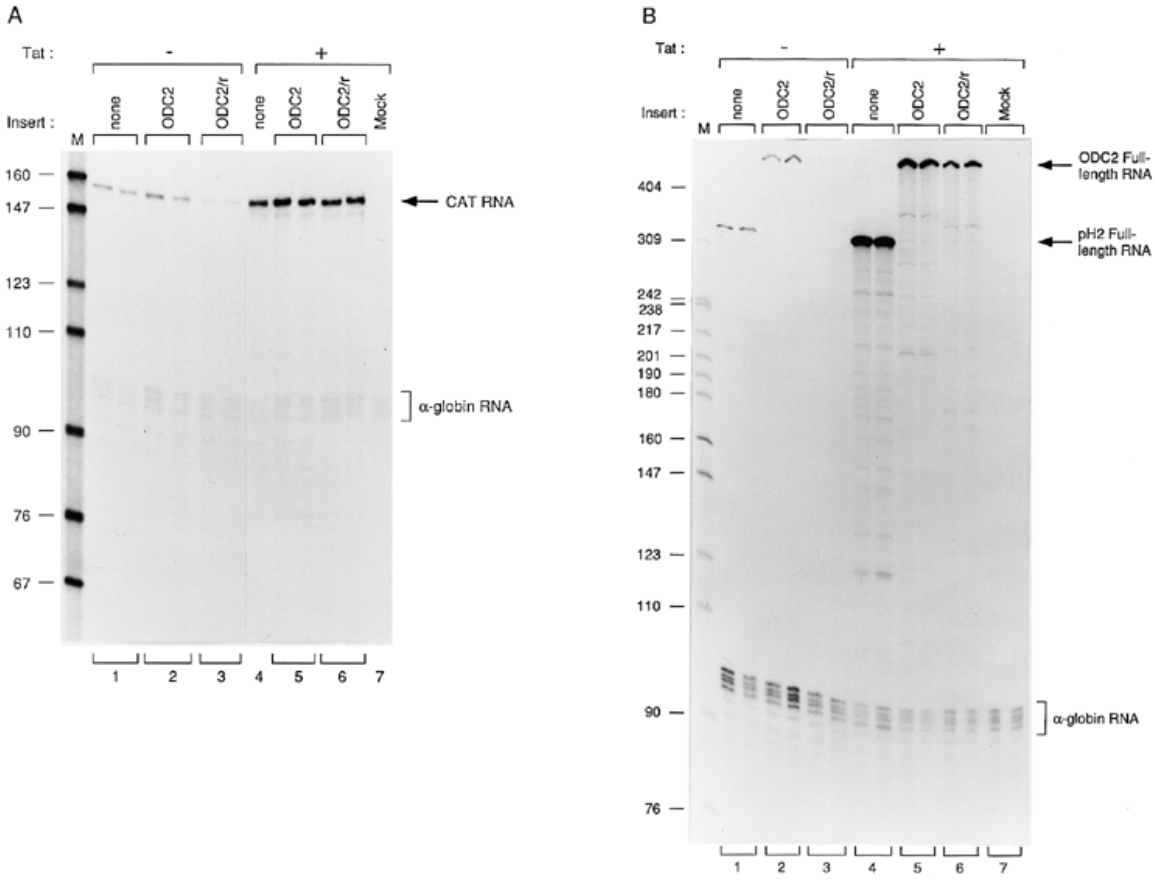

Figure 4. Analysis of the ODC attenuator sequence. COS cells were transfected with pH2 (lanes 1 and 4), ODC2 (lanes 2 and 5), ODC2/r (lanes 3 and 6), with or without pCMVTat as indicated, or with pCMVTat alone (lane 7). RNA was assayed by RNase protection using (**A**) PCNA-CAT riboprobe and (**B**) homologous riboprobes.

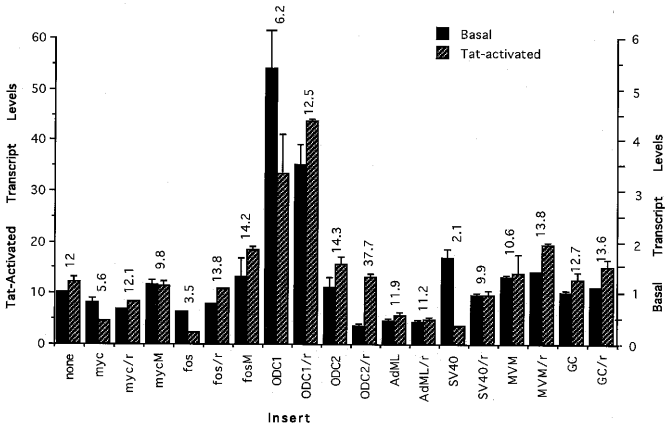

Figure 5. Quantitation of readthrough transcription into the CAT gene. Experiments such as those of Figures 2–4 were quantified by measuring the CAT RNA signal obtained with the PCNA-CAT riboprobe and normalizing to the α-globin signal. Transcript levels were assessed from at least four transfections and are expressed relative to cells transfected with pH2 in the absence of Tat. Error bars denote standard deviations. Solid columns: basal readthrough transcription in cells transfected with the indicated construct relative to pH2. Hatched columns: readthrough transcription in cells co-transfected with the indicated construct together with pCMV-Tat relative to pH2 alone. Note the 10-fold difference in scales. The numbers above each pair of bars gives the fold transactivation by Tat.

homologous probes as described above (Fig. 1B). Unlike the parental plasmid pH2 (15), no short RNA species corresponding to an attenuated fragment was detected in compensation for the reduction in transcript level due to the presence of the ODC2/r insert (Fig. 4B, lanes 3).

DISCUSSION

With the identification of cellular factors recruited by Tat as an objective, we endeavored to extend the finding that Tat can promote transcriptional readthrough of an artificial terminator *in vitro* (12,39). To this end, we inserted downstream of the TAR element in the HIV-1 LTR various DNA fragments that have been reported to act as attenuators. The resulting constructs were transfected into Cos-1 cells in the presence or absence of a plasmid expressing the first exon of Tat. Since most of these sequences had previously been characterized as attenuators only in cell-free systems, our initial goals were 2-fold: first, to observe their effect on transcription *in vivo*; and, second, to test the ability of Tat to impel readthrough of the attenuators. We expected to observe varying degrees of attenuation *in vivo* with the different attenuators, and hoped that varying degrees of Tat-promoted elongation through the attenuator sequences would throw light on the factors and mechanisms involved.

The sequences studied had all been reported to serve as attenuators of cell-free transcription but most of them had not been tested when placed downstream of the HIV LTR. Cell-free transcription

experiments confirmed that several of these subcloned sequences led to the formation of the expected attenuated RNAs initiated from the HIV promoter. Unfortunately, we were unable to determine whether Tat overcame attenuation *in vitro* because the specialized conditions required to induce the appearance of the attenuated transcripts proved to be incompatible with the Tat response. In transient expression assays, on the other hand, none of the sequences reported to function *in vitro* behaved as an attenuator although many of the inserts markedly affected transcription as summarized in Figure 5. Ironically, evidence for attenuation *in vivo* was obtained only in the case of ODC2/r, in which the sequence is inverted relative to the orientation reported to function as an attenuator *in vitro*. Moreover, Tat was strikingly effectively in overcoming the inhibitory effect of ODC2/r on RNA readthrough *in vivo*. It should be noted, however, that short RNA transcripts mapping to the expected site of premature termination were not detected (data not shown), perhaps because they are labile *in vivo*.

Notwithstanding the disappointing behavior of these sequences as attenuators *in vivo*, the placement of inserts downstream of TAR exerted remarkably variable effects upon the basal readthrough levels of CAT RNA and on transactivation by Tat (Fig. 5). In comparison with the parent pH2 plasmid, the effects of the inserts can be grouped as follows: (i) decreased basal transcription but no effect on transactivated levels (ODC2/r); (ii) decreased basal and transactivated levels (AdMLP, AdMLP/r, myc, fos); (iii) unchanged basal and transactivated levels (GC, MVM, ODC2); (iv) increased basal and transactivated levels (ODC1, ODC1/r); and (v) increased basal levels and decreased transactivated levels (SV40).

The ODC2/r insert was the only one to display attenuator properties: it yielded the greatest degree of inhibition of downstream readthrough RNA (~3-fold), while ODC2 had no significant effect. In the presence of Tat, neither insert had a dramatic effect. Consequently, ODC2/r evinced exceptionally high transactivation whereas ODC2 transactivated to the same extent as pH2. Thus ODC2/r behaved as expected for a true Tat-suppressible attenuator, even though runoff transcription *in vitro* did not give rise to a detectable attenuated band or to decreased RNA yield (data not shown). The ODC2/r sequence contains a cluster of 30 adenine residues within a 35 nt stretch. Adenine rich sequences have been shown to be intrinsic attenuators in prokaryotes (17).

The adenovirus inserts, AdML and AdML/r, brought about an ~2-fold decrease in both basal and Tat-transactivated readthrough transcription (Fig. 2B). The orientation-independence of the effect suggests that a DNA element embedded within the cloned AdML fragment may function as a general transcriptional downregulator. While transcription repressors are commonly thought to function during initiation, it is conceivable that DNA binding factors may function downstream to inhibit transcription and depress RNA levels. Attenuator sequences have been characterized as RNA elements due to their orientation specificity *in vitro* and by base substitutions that destabilize the RNA stem–loop structure leading to reduced attenuation activity (20). A DNA element, the inducer of short transcripts (IST) directs formation of poorly elongating transcription complexes (16) and it is possible that a functionally equivalent sequence is present within the AdML insert.

The myc and fos inserts were mildly inhibitory in the absence of Tat and distinctly inhibitory in its presence. Consequently the fold transactivation by Tat was reduced by these inserts. These effects were not observed when the inserts were reversed, and were largely if not entirely due to the presence of a donor splice site as evidenced by RNase protection and mutagenic data. Our observation that the short RNA species from the myc and fos transfections arise from a splicing event and not premature termination complicates the interpretation of some previous studies which map the attenuation sites in the myc gene adjacent to the exon 1/intron 1 border (27,28), but other experiments imply that promoter-proximal pausing can occur even when the splice site is deleted (51).

Surprisingly, neither the MVM attenuator nor the synthetic terminator sequence GC, both characterized as effective attenuators *in vitro*, gave substantial effects in our transfection assays. Although the GC insert is identical to the one used by Graeble and co-workers (12), the TAR and GC elements are closer to one another in our constructs than in theirs (20 nt downstream from the 3′ end of the TAR stem–loop compared to 120 nt in pMAG-10). This difference is unlikely to provide an explanation for the discrepancy, however, since a short RNA corresponding to the 3′ end of the termination sequence was observed in the cell-free transcription assay in the presence of high salt (data not shown).

The ODC1 insert enhanced transcription independent of orientation and of the presence of Tat, suggesting that it contains a stimulatory DNA element. The high basal levels obtained for the ODC1 inserts may be explained by the two upstream stimulatory factor (USF) consensus binding sequences located within the ODC1 attenuator sequences (45).

The SV40 insert gave rise to a modest increase in basal transcription yet a significant decrease in Tat-activated transcription. Transactivation was reduced to its lowest level (2-fold), implying that Tat-modified transcription complexes may be less able than normal complexes to pass through the SV40 attenuator. Alternatively, the SV40 sequence may render transcription complexes formed at the HIV-1 promoter more refractory to Tat. Earlier studies indicated that the secondary structure of the SV40 RNA attenuator element influences the elongation properties of the polymerase *in vitro* (47,52). Therefore it is possible that Tat binding may stabilize the attenuation conformation or block access of a protein required to maintain the readthrough conformation. Use of the GCG program ‘MFold’ to generate alternate RNA conformations of TAR followed by the SV40 attenuator sequence supports this conjecture. Proper folding of the TAR element allows the creation of the SV40 attenuation conformation as described by Hay and Aloni, but the 3′ stem of TAR can hybridize with downstream nucleotides to disrupt this attenuation conformation. Tat’s interaction with TAR presumably stabilizes the TAR structure, thus allowing for formation of the SV40 attenuator conformation and resulting in a greatly reduced Tat effect.

Why did we not observe attenuation *in vivo*? Experimentally, attenuation is inferred from the detection of short, prematurely-terminated transcripts or by monitoring a decrease in readthrough transciption levels. We employed both methods to assay for attenuation in our constructs, raising the possibility that the inserted sequences do not function as attenuators *in vivo*. Before drawing this conclusion, however, several caveats must be considered. First, the failure to detect short transcripts might be related to the kinetics of transcriptional pausing and RNA stability. We analyzed the steady-state levels of RNA accumulated during an extended period after transfection: while RNA polymerase complexes presumably stall throughout this time, it is likely that not all prematurely released transcripts are sufficiently stable to persist in the cell. Although Blair *et al*. (53) were able to detect such truncated RNAs using a large insert specifying a highly organized structural element (the EMCV

IRES), other RNA sequences 3′ of TAR may be degraded. Indeed, 3′ processing leaving a hairpin ending at the 3′ base of the TAR structure has been observed both *in vivo* and *in vitro* (15), and such transcripts were detected in all of the transfection experiments reported here (see Fig. 3 for example). Second, special conditions may be required to enhance the rate of attenuation in the cell, as in cell-free systems where detergent or high salt concentrations are routinely used to induce the appearance of attenuated transcripts. For example, treatment of HL-60 cells with retinoic acid, an inducer of differentiation, results in a downregulation in full-length RNA levels and a 10-fold increase in attenuated RNAs (21). Appropriate conditions might be missing in the transfection assays used here. Third, the nature of the promoter may have an influence. Although several of the inserts gave rise to short RNAs of the expected sizes *in vitro* when placed downstream of the HIV promoter (data not shown), attenuation may be largely promoter dependent in the cell (51). A comparison of our findings with those of Wright *et al.* (39) is consistent with this possibility. When these authors placed the TAR element downstream of the myc P2 promoter, they detected an accumulation of polymerase complexes upstream of the myc attenuator sequence that was alleviated by transfection of Tat. When the myc attenuator sequence was installed downstream of the HIV promoter, on the other hand, we failed to detect attenuation.

Finally, our observations underscore the role of downstream sequences in determining transcription levels. Both basal and Tat transactivated levels varied over a >20-fold range depending on the nature of the inserted downstream sequences. Certain DNA binding proteins function as architectural components to load or recruit transcription factors and basal components. The DNA sequences within the subcloned fragments may also block access of elongation factors, such that RNA levels are altered. Recently, local chromatin structure has been identified as an important factor affecting transcription levels (54). The variable transcription levels of our chimeric templates are worthy of further investigation from these perspectives.

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