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Functional Analysis of a Stable Transcription Arrest Site in the First Intron of the Murine Adenosine Deaminase Gene

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Transcription arrest plays a role in regulating the expression of a number of genes, including the murine adenosine deaminase (ADA) gene. We have previously identified two prominent arrest sites at the 5′ end of the ADA gene: one in the first exon and one in the first intron (J. W. Innis and R. E. Kellems, Mol. Cell. Biol. 11:5398–5409, 1991). Here we report the functional characterization of the intron 1 arrest site, located 137 to 145 nucleotides downstream of the cap site. We have determined, using gel filtration, that the intron 1 arrest site is a stable RNA polymerase II pause site and that the transcription elongation factor SII promotes read-through at this site. Additionally, the sequence determinants for the pause are located within a 37-bp fragment encompassing this site (+123 to +158) and can direct transcription arrest in an orientation-dependent manner in the context of the ADA and adenovirus major late promoters. Specific point mutations in this region increase or decrease the relative pausing efficiency. We also show that the sequence determinants for transcription arrest can function when placed an additional 104 bp downstream of their natural position.

Premature transcription arrest is a factor in the control of expression of many prokaryotic, eukaryotic, and viral genes (for a comprehensive review, see reference 61). Examples of eukaryotic genes include the cellular proto-oncogenes human and murine c-*myc* (4, 13, 30, 43), human L-*myc* (33), murine c-*myb* (2, 63), rat, murine, and hamster c-*fos* (15, 34), the human and murine adenosine deaminase (ADA) genes (6–8, 26, 35, 38, 45), the human histone H3.3 gene (50), the human epidermal growth factor receptor gene (18), and the *Drosophila* heat shock genes (53, 54) as well as several other *Drosophila* genes (54). Some viral transcription units also contain elongation controls; examples include human immunodeficiency virus (28), simian virus 40 (22, 23), adenovirus type 2 (20, 39, 42, 64), polyomavirus (60), and minute virus of mice (1, 51). Two mechanisms can lead to premature transcription arrest. First, the polymerase may stop but not be released from the template, resulting in a pause. This is the case for the *Drosophila* heat shock genes, in which sequences in the promoter are capable of setting up a paused polymerase complex (36). Upon heat shock induction, the block to elongation is overcome, resulting in the production of full-length transcripts. One transcription elongation factor, SII (3, 46, 48, 56), has been shown to promote read-through of several pause sites, including those in the histone H3.3 gene (59) and the adenovirus major late promoter transcription unit (46, 48). The second mechanism involves RNA polymerase II dissociation from the template accompanied by the release of the nascent transcript, or premature termination. In the case of human immunodeficiency virus, prematurely terminated transcripts are produced by the inducer of short transcripts, which is located between −3 and +82 relative to the cap site (62). These transcripts can also be detected in vivo in the absence of Tat (28).

In some cases, multiple elongation blocks have been described within a gene. For example, in the murine c-*fos* gene, a negative regulatory element (the FIRE, or *fos*-intragenic regulatory element) which lies in the first exon has been identified (34). This block can be released by the titration of a putative factor, as shown by the release of the block in a p-*fos*–*lacZ* test construct upon coinjection of the FIRE sequence into rat fibroblasts (34). Within the first intron of the c-*fos* gene, a block to elongation which is calcium dependent has been found in murine macrophages. Functional analysis of this block has revealed that a 103-nucleotide (nt)-long intron 1 sequence is sufficient for obtaining the block in vitro (41). Similarly, in the c-*myc* gene, the site of transcription arrest has been mapped to two locations, one in the first exon (T_I) and one in the first intron (T_{II}) (5, 13, 30, 37). Site T_I is preceded by dyad symmetry and contains a uridine stretch (5). If this signal is similar to the rho-independent termination signals in prokaryotes, formation of the stem-loop in the nascent transcript leads to a polymerase pause in the nearby uridine tract, where dissociation of the template, polymerase, and nascent RNA is thought to occur (19). Gel filtration experiments revealed that the T_{II} transcripts are released from the template and thus represent transcription termination (37). It has also been found that an oligonucleotide specifying the human c-*myc* T_{II} sequence is sufficient to mediate transcription termination in a HeLa nuclear extract (37). Interestingly, in the case of the human c-*myc* gene, recent evidence has shown that sequences upstream of +47 are sufficient to confer promoter-proximal pausing of polymerases (32). In this case, it is thought that modification of polymerase complexes at the promoter-proximal pause site may determine whether polymerases can read through the downstream intrinsic sites of termination.

Our model system for studying transcription attenuation is the ADA gene. ADA activity and mRNA levels vary by as much as 10,000-fold among mouse tissues and cell types (8, 35). Nuclear run-on experiments revealed a block to transcription elongation at the 5′ end of the gene in tissues with low levels of ADA expression (6, 8, 38). *Xenopus* oocyte

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injection experiments using either the human (6, 7) or murine (45) ADA genes revealed the accumulation of short transcripts with 3′ ends mapping to the first exon. An in vitro transcription system for the murine ADA gene which reproduced the oocyte findings was developed (26, 27). Depending on the in vitro conditions, it was also possible to observe a prominent transcription arrest site in the first intron. Both the exon 1 and intron 1 transcription arrest sites lie within the region of elongation block defined by the nuclear run-on studies and may have a role in the tissue-specific regulation of ADA gene expression.

In this study, we characterize the intron 1 transcription arrest site of the murine ADA gene. We have shown that transcripts mapping to this site are the result of polymerase pausing and that the transcription elongation factor SII can promote read-through of this site. We have determined that a 37-bp fragment encompassing this site is sufficient to encode the intron 1 pause site in the context of the ADA or adenovirus major late promoter. Single-point mutations in this region are able to increase or decrease (by as much as 60%) the relative arrest efficiency. These sequences function in an orientation-dependent manner and when placed an additional 104 bp downstream of their natural position.

MATERIALS AND METHODS

Plasmid constructions. Generation of the parental ADA subclones (0.3ADA and 0.4ADA) which contain positions −154 to +168 and −154 to +206 of the ADA gene has been described previously (45). These constructs contain the ADA promoter, first exon (122 bp), and part of the first intron in pTZ19R (Pharmacia). All numbers refer to ADA sequences, on which +1 is the transcription start site.

To create 5′UT+8, 5′UTrev, and 5′UTpTZ, a unique *Xho*I site was created in 0.3ADA at +3 by *Dde*I (+3) partial digestion of the plasmid, mung bean nuclease digestion, and religation. The *Xho*I-*Nco*I fragment containing the 5′ untranslated region (+3 to +92) was removed, and both vector and insert were filled in with DNA polymerase I. This region was then replaced by the same fragment in the native (5′UT+8) or opposite (5′UTrev) orientation or by a 95-bp pTZ19R *Alu*I fragment (5′UTpTZ). The internal deletion mutant 81/98 (numbers refer to inclusive deletion) were created by linearization at the *Nco*I site (+92) in 0.4ADA followed by BAL 31 digestion, a fill-in reaction, and religation. The internal deletion mutants 101/111 and 101/117 were created by linearization at the *Bsm*I site (+102) in 0.4ADA followed by S1 nuclease digestion, a fill-in reaction, and religation.

Mutants −154/+92, −152/+102, and −154/+158 were created by digestion of 0.4ADA at the *Hin*dIII site in the downstream polylinker region and the internal *Nco*I (−154 to +92), or *Bsm*I (−152 to +102), or *Sma*I (−154 to +158) site. Plasmids were filled in and religated. Mutants −154/+114 and −154/+138 were constructed by using a polymerase chain reaction (PCR) strategy involving a primer from +3 to +23 of ADA as an upstream primer and a primer from +90 to +114 (−154/+114) or a primer from +113 to +138 (−154/+138) as a downstream primer. PCR products were subcloned into an ADA promoter vector. Mutants G142T, G145T, C146A, and C147A were created by recombinant PCR (25) with mutant oligonucleotides. For example, in mutant G142T, the G at +142 was changed to a T.

To create the series of insertion mutants, the unique *Nco*I site at +92 of 0.4ADA was used. Digestion and fill-in resulted in the parent +4 mutant. Insertion of a 35-bp *Msp*I-digested pBR322 filled-in fragment led to the parent +35 mutant. Insertion of either one or two copies of an *Nla*IV fragment from the ADA gene (+86 to +138) in the forward orientation resulted in the parent +52 and parent +104 mutants, respectively.

PCR using the 0.4ADA plasmid with an upstream primer from +123 to +143 and a downstream pTZ19R reverse primer (Stratagene; as described above) followed by *Sma*I digestion was the technique used to generate the +123 to +158 fragment of the ADA first intron. This fragment was inserted in either orientation at the filled-in *Nco*I site at +92 of ADA (0.4ADA) to create ADA + forward and ADA + reverse.

The Adeno plasmid contains positions −260 to +33 relative to the transcription start site (a *Xho*I-*Pvu*II fragment) of the adenovirus major late promoter as a replacement of the *Xho*I-*Sma*I polylinker region of Bluescript KS (+). The ADA fragment from +123 to +158 was inserted in either orientation at the downstream filled-in *Xba*I site to create Adeno + forward and Adeno + reverse. A primer specific for transcripts from this adenovirus major late promoter was used in primer extension assays (+31 to +49; 5′-GAACT AGTGGATCCCCCCTG-3′).

All plasmids were purified twice by CsCl density equilibrium centrifugation and checked by restriction digestion. Mutations were confirmed by dideoxy sequencing (U.S. Biochemical Corp.) of the plasmid DNA, using appropriate primers.

Preparation of HeLa cell nuclear extracts and in vitro transcription assay. Suspension cultures of HeLa S3 cells were maintained in RPMI 1640 supplemented with 5% fetal calf serum. Four- to six-liter cultures of cells were harvested during log-phase growth (a density of $\sim 5 \times 10^5$ cells per ml) and used to prepare a nuclear extract as described previously (12). Depending on the extract preparation, the protein concentration varied from 4 to 7 mg/ml. Aliquots of 200 μl were frozen in liquid nitrogen and stored at −70°C until use. In vitro transcription was performed as described previously (26), using 270 ng of template DNA per 20-μl reaction.

Gel filtration of transcription reactions. A 10-ml-bed-volume column was formed from Sepharose CL-4B (Sigma) equilibrated at 4°C in transcription buffer supplemented with 80 μg of heparin per ml and 20 μg of acetylated bovine serum albumin per ml. A scaled-up (5×) transcription reaction was carried out with 0.3ADA DNA linearized at a downstream *Hin*dIII site, and the mixture was filtered through the column at 4°C. Fractions of ~300 μl were collected, and those containing radioactivity were divided into two aliquots. One aliquot was stored at 4°C, and the other was incubated at 30°C for 10 min in the presence of 500 μM nucleoside triphosphates (NTPs) (unlabeled). All nucleic acids were extracted with an equal volume of phenol-chloroform. Precipitation and gel electrophoresis were done as described above. In experiments with SII, several nanograms of protein from either a purified fraction of HeLa SII or recombinant bacterial SII were used; both were kindly provided by M. Sawadogo. Column fractions were divided into thirds; one was incubated with nothing, one was incubated with 500 μM NTPs, and one was incubated with 500 μM NTPs plus SII at 30°C for the times indicated.

RNase protections. RNase protections were performed as described previously (26), with the following modifications. (i) RNA probe templates consisted of either the plasmid DNA linearized near the ADA transcription start site or template-specific PCR products. The PCR products were

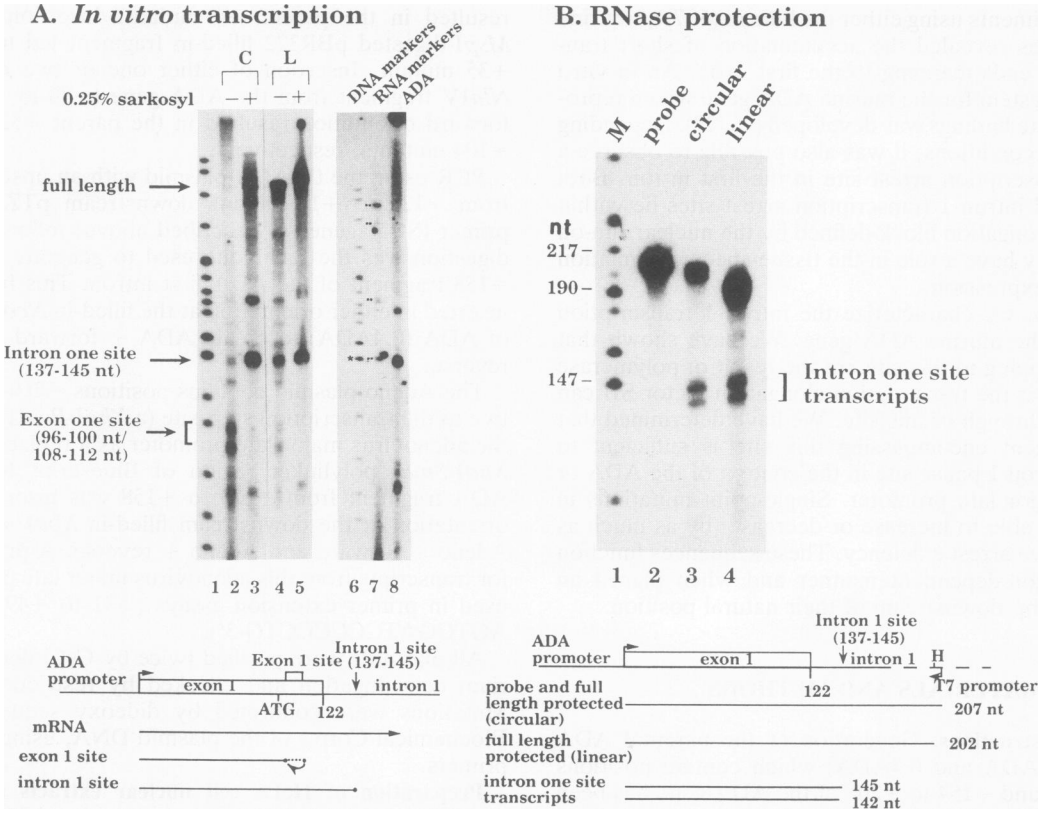


FIG. 1. Occurrence of transcription arrest at the intron 1 arrest site in the presence of Sarkosyl on linear or circular templates. (A) In vitro transcription reactions were performed as described in Materials and Methods with circular (C) or linear (L) ADA templates (0.4ADA). Where indicated, at 1 min following addition of NTPs, 0.25% (wt/vol) Sarkosyl was added and the reaction was continued for 15 min at 30°C. RNA was extracted, separated on 6% polyacrylamide–7 M urea gels, and subjected to autoradiography. The exon 1 and intron 1 site transcripts are indicated. Below is a schematic of the 5′ end of the murine ADA gene; the locations of these transcripts are indicated. Lanes: 1, marker DNA (pBR322 *Msp*I); 2, RNA products from a transcription reaction with a circular template (0.4ADA); 3, identical to lane 2 except that 0.25% Sarkosyl was added 1 min after addition of NTPs; 4, RNA products from a transcription reaction with a linearized template (0.4ADA *Pvu*II; full length = 435 nt); 5, identical to lane 4 except that 0.25% Sarkosyl was added 1 min after addition of NTPs; 6, marker DNA (pBR322 *Msp*I; * = 147 bp); 7, RNA markers, indicated with double dots (145, 208, 390, and 481 nt); 8, RNA products from a transcription reaction with a circular template (0.4ADA) in which 0.25% Sarkosyl was added 1 min after the addition of NTPs. Radiolabeled RNA size markers were electrophoresed next to the intron 1 site transcripts. The 145-nt RNA marker comigrates with the intron 1 site transcripts as shown in lanes 7 and 8. (B) RNase protection. Unlabeled RNA from a transcription reaction with circular or linear DNA (performed with the addition of Sarkosyl) was hybridized to a uniformly labeled RNA probe complementary to +3 to +168 of the ADA gene (+1 = transcription start site). Unhybridized RNA was digested by RNase, and protected products were extracted, separated by electrophoresis on a 6% polyacrylamide–7 M urea gel, and visualized by autoradiography. The intron 1 site transcripts are indicated, and the expected products are diagrammed below. Lanes: 1, marker DNA (pBR322 *Msp*I); 2, RNA probe; 3, protected products after hybridization with RNA from a transcription reaction with a circular template; 4, protected products after hybridization with RNA from a transcription reaction with a linearized template. Abbreviation: H, *Hin*dIII.

generated by using an upstream ADA primer (+3 to +23) and a downstream vector-specific reverse primer with 1 ng of plasmid DNA. The products thus contained ADA sequences, polylinker sequences, and the T7 polymerase promoter. (ii) The unlabeled RNA was prepared and redissolved in 200 μl of 1× DNase I digestion buffer (final concentrations, 50 mM Tris (pH 7.5) and 10 mM $MnCl_2$). DNase I digestion (RQ1 DNase; Promega) followed, using 3 U at 37°C for 30 min. The nucleic acids were extracted and precipitated at −70°C with ethanol and 40,000 to 60,000 cpm of the gel-purified RNA probe (constant within an experiment) or 300,000 cpm of the non-gel-purified RNA probe. Hybridization and digestion by RNase were done as described previously (26).

Primer extensions. Unlabeled RNA was prepared as described above and precipitated with 500,000 cpm of a 5′-^{32}P-labeled primer complementary to +31 to +49 of the adenovirus major late promoter transcript (described above). The sample was redissolved in a solution containing 10 mM Tris (pH 7.5), 0.3 M NaCl, and 1 mM EDTA. After denaturation at 95°C for 5 min, hybridization proceeded for 3.5 h at 23°C. Primer extension occurred at 42°C for 2 h in a 90-μl volume containing 55 mM Tris (pH 7.5), 11 mM dithiothreitol, 8.8 mM $MgCl_2$, 33 mM NaCl, 550 μM each dNTP, 400 μg of actinomycin D per ml, and 10 U of avian myeloblastosis virus reverse transcriptase (Life Sciences, Inc.). Upon precipitation, the reaction products were prepared for gel electrophoresis as described above.

RESULTS

Transcriptional arrest at the intron 1 site occurs in the presence of 0.25% Sarkosyl on circular or linear templates. Previous research has used *Xenopus* oocyte injection experiments to characterize the transcription arrest processes occurring at the 5′ end of the murine (45) and human (6, 7)

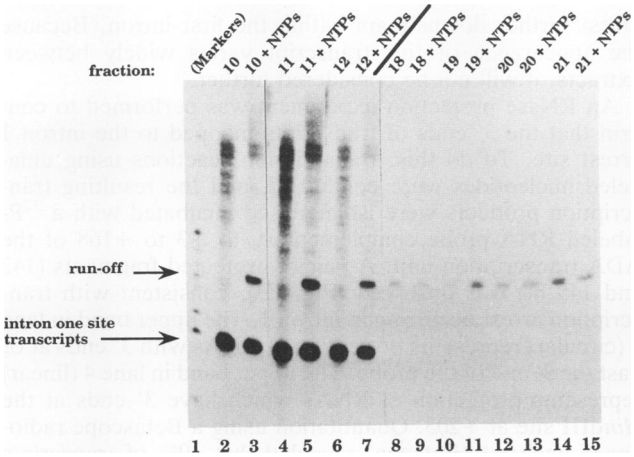


FIG. 2. Gel filtration of transcription complexes, indicating that the intron 1 site represents a stable transcription pause site. A complete in vitro transcription reaction was filtered through a Sepharose CL-4B column. Fractions (300 μl) containing radioactivity were collected and divided in half. For one half, the nucleic acids were extracted and precipitated (even-numbered lanes). The other half was chased with 1 mM NTPs for 10 min at 30°C, and then the nucleic acids were extracted and precipitated (odd-numbered lanes). Early fractions (lanes 2 to 7) contain active transcription complexes (note prominent intron 1 site transcripts in both lanes and full-length runoff [205 nt] in chase lanes), and later fractions (lanes 8 to 15) contain free, terminated RNA (note runoff RNA in these lanes). Positions of the intron 1 site transcripts and full-length runoff are labeled. Lane 1 contains DNA markers (pBR322 *Msp*I).

ADA genes. An in vitro transcription assay using HeLa cell nuclear extracts was developed which reproduced the oocyte findings and revealed the presence of transcription arrest sites in exon 1 and intron 1 of the murine ADA genes (27, 26). The transcription products of a reaction using circular templates are shown in Fig. 1A (lane 2). Transcripts mapping to exon 1 included those in the size range of 96 to 100 nt (previously termed site I transcripts) and those in the size range of 108 to 112 nt (previously termed site II transcripts). Pulse-chase experiments showed that the 96- to 100-nt transcripts are processed products of 108- to 112-nt precursor transcripts (26). The addition of Sarkosyl (0.25%) 1 min after the initiation of transcription resulted in the disappearance of transcripts mapping to exon 1 and the increased abundance of 137- to 145-nt transcripts that map to intron 1 (Fig. 1A, lane 3; Fig. 1B [see below]). This finding illustrates that the exon 1 transcription arrest process is sensitive to Sarkosyl. Sarkosyl at this concentration has been shown to block initiation but not elongation (20, 21), thus limiting the reaction only to those polymerase molecules which initiate during the first minute. When transcription reactions were performed with linearized templates (Fig. 1A, lane 4), a prominent collection of transcripts (137 to 145 nt) that map to intron 1 was observed. These findings show that transcription arrest in intron 1 is favored over transcription arrest in exon 1 in transcription reactions using linearized templates. The addition of Sarkosyl (0.25%) 1 min after the initiation of transcription reactions resulted in the disappearance of transcripts arrested in exon 1 and a further increase in the abundance of 137- to 145-nt transcripts

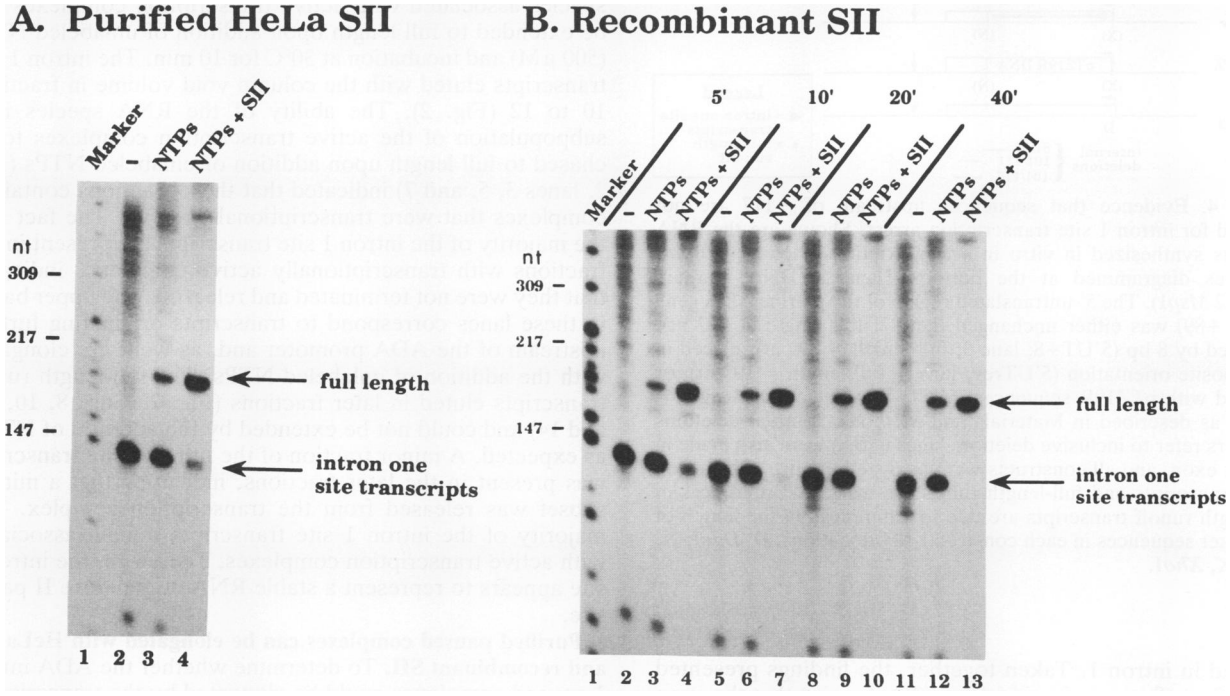


FIG. 3. Elongation of purified paused complexes with HeLa SII and recombinant SII. (A) A complete in vitro transcription reaction was filtered through a Sepharose CL-4B column. Fractions (300 μl) containing radioactivity were collected and divided into three aliquots. For one part, the nucleic acids were extracted and precipitated (lane 2). A second aliquot was chased with 500 μM NTPs for 20 min at 30°C, and then the nucleic acids were extracted and precipitated (lane 3). A third aliquot was chased with 500 μM NTPs plus purified HeLa SII for 20 min at 30°C, and then the nucleic acids were extracted and precipitated (lane 4). Lane 1 contains DNA markers (pBR322 *Msp*I). The intron 1 site transcripts and full-length runoff (205-nt) transcripts are indicated at the right. (B) Same as panel A except that peak fractions were pooled and, where indicated, incubated with 500 μM NTPs plus recombinant SII for the times indicated. Lane 1 contains DNA markers (pBR322 *Msp*I).

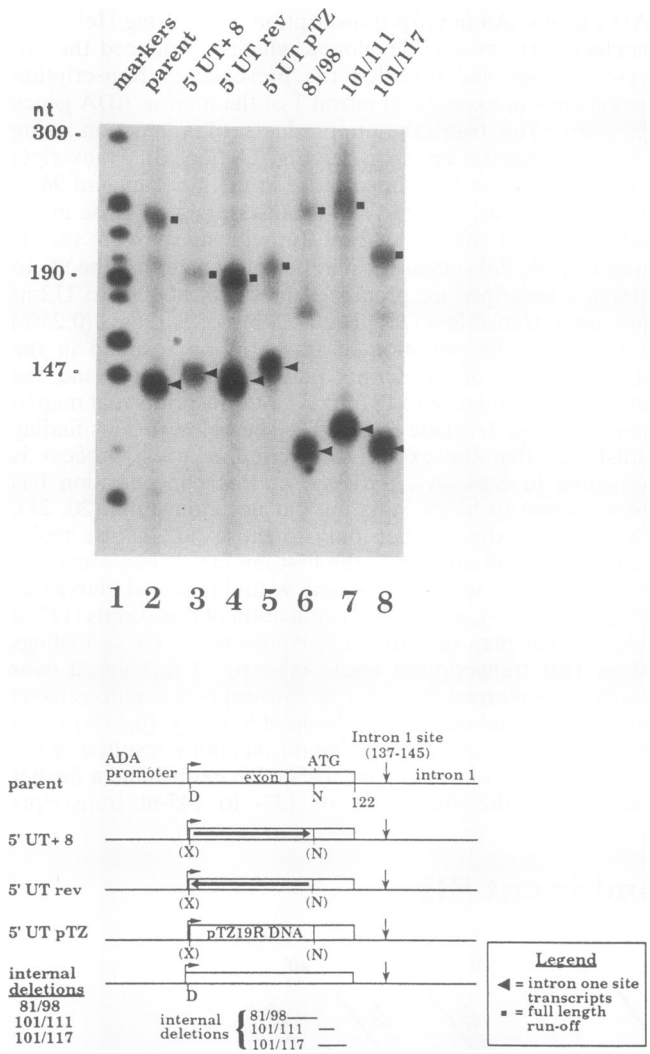


FIG. 4. Evidence that sequences upstream of +117 are not required for intron 1 site transcription arrest. Shown are the RNA products synthesized in vitro in a transcription reaction with the templates diagrammed at the bottom. Lane 1, DNA markers (pBR322 *Msp*I). The 5′ untranslated region of the murine ADA gene (+3 to +89) was either unchanged (lane 2; full length = 227 nt), increased by 8 bp (5′UT+8; lane 3; full length = 195 nt), placed in the opposite orientation (5′UTrev; lane 4; full length = 195 nt), or replaced with pTZ19R sequences (5′UTpTZ; lane 5; full length = 200 nt) as described in Materials and Methods. Internal deletions (numbers refer to inclusive deletion; lanes 6 to 8) were also made in the first exon, and all constructs were analyzed in vitro. The intron 1 site transcripts and full-length runoff are marked. Differences in full-length runoff transcripts are due to differences in the length of polylinker sequences in each construct. Abbreviations: D, *Dde*I; N, *Nco*I; X, *Xho*I.

arrested in intron 1. Taken together, the findings presented in Fig. 1 confirm previous findings (26) showing that the exon 1 and intron 1 transcription arrest sites differ with regard to sensitivity to Sarkosyl and template topology. The synthesis of both transcripts is sensitive to α-amanitin (data not shown and reference 26), and neither is formed by a cleavage mechanism when longer RNAs are added back to extracts (26). An additional transcript approximately 190 nt in length also appears upon addition of Sarkosyl and corresponds to arrest further downstream within the first intron. Because the appearance of this transcript varies widely between extracts, it will not be considered further.

An RNase protection experiment was performed to confirm that the 3′ ends of transcripts mapped to the intron 1 arrest site. To do this, transcription reactions using unlabeled nucleotides were conducted, and the resulting transcription products were isolated and incubated with a ^{32}P-labeled RNA probe complementary to +3 to +168 of the ADA transcription unit. A pair of protected fragments (142 and 145 nt) was observed (Fig. 1B), consistent with transcription arrest occurring in intron 1. The upper band in lane 3 (circular) represents protection of RNAs with 3′ ends at or past the 3′ end of the probe. The upper band in lane 4 (linear) represents protection of RNAs which have 3′ ends at the *Hin*dIII site at +205. Quantitation using a Betascope radioimage analyzer (Betagen) revealed that 40% of transcripts arrested in a circular-template reaction and that 30% of the transcripts arrested in a linear-template reaction. Thus, the intron 1 site transcripts accumulate in vitro, using circular or linear templates, upon inclusion of Sarkosyl in the transcription reaction. The use of Sarkosyl in this regard facilitates the analysis of transcription arrest in intron 1 by inhibiting transcription arrest in exon 1.

The intron 1 arrest site is a transcription pause site. To examine whether the intron 1 site transcripts were formed by transcription pausing or termination (release of the transcript), we separated the transcription complexes from the free, terminated RNA species by gel filtration. Paused and active transcription complexes elute in the void volume, whereas free, terminated RNA transcripts are retarded by the column matrix (37). In theory, after filtration, RNA species associated with active transcription complexes can be extended to full length upon addition of unlabeled NTPs (500 μM) and incubation at 30°C for 10 min. The intron 1 site transcripts eluted with the column void volume in fractions 10 to 12 (Fig. 2). The ability of the RNA species in a subpopulation of the active transcription complexes to be chased to full length upon addition of unlabeled NTPs (Fig. 2, lanes 3, 5, and 7) indicated that these fractions contained complexes that were transcriptionally active. The fact that the majority of the intron 1 site transcripts are present in the fractions with transcriptionally active complexes indicated that they were not terminated and released. The upper bands in these lanes correspond to transcripts originating further upstream of the ADA promoter and, as well, are elongated with the addition of unlabeled NTPs. The full-length runoff transcripts eluted in later fractions (Fig. 2, lanes 8, 10, 12, and 14) and could not be extended by the addition of NTPs, as expected. A minor fraction of the intron 1 site transcripts was present in the later fractions, indicating that a minute subset was released from the transcription complex. The majority of the intron 1 site transcripts remain associated with active transcription complexes. Therefore, the intron 1 site appears to represent a stable RNA polymerase II pause site.

Purified paused complexes can be elongated with HeLa SII and recombinant SII. To determine whether the ADA intron 1-paused complexes could be elongated by the transcription elongation factor SII (46, 48, 56), we added either a purified fraction of SII (Fig. 3A) or recombinant SII (Fig. 3B) to column-fractionated transcription complexes. Column fractions containing intron 1-paused complexes were divided into three aliquots; one was incubated with buffer alone, one was incubated with buffer plus 500 μM NTPs, and one was incubated with buffer plus 500 μM NTPs and SII. A 20-min

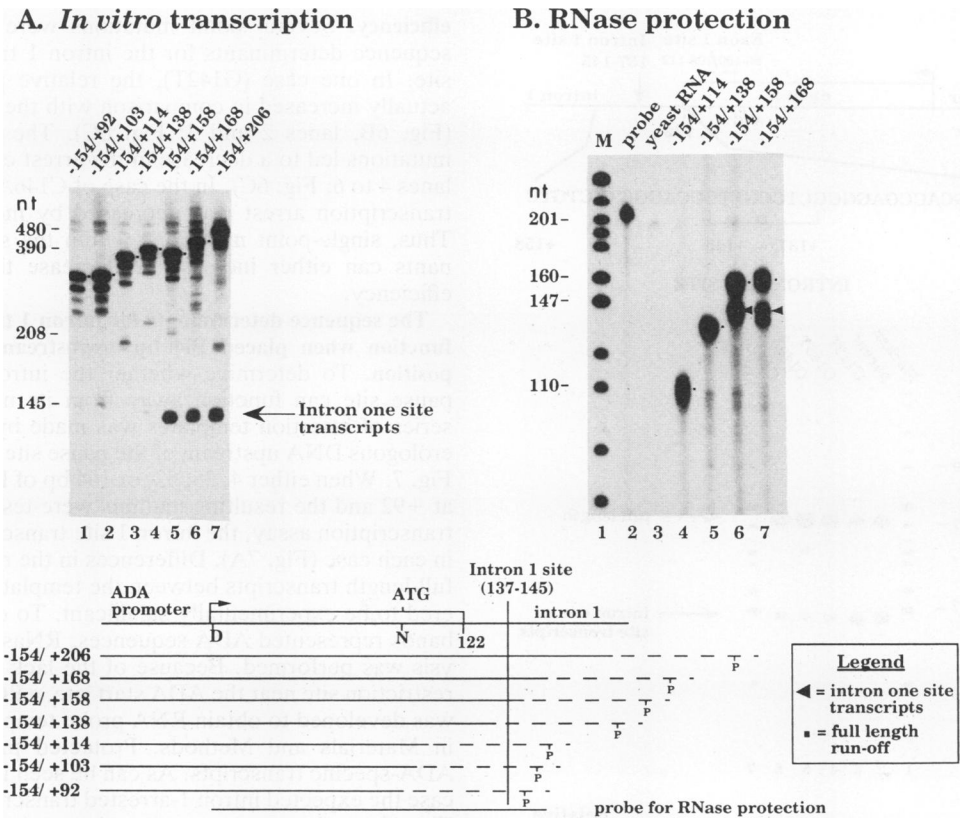


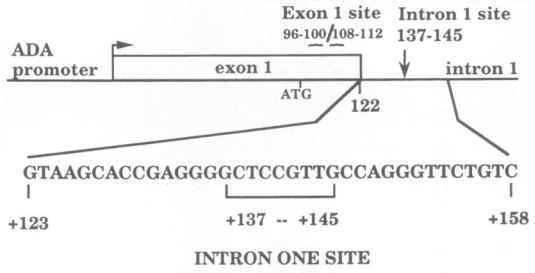
FIG. 5. Evidence that sequences downstream of +158 are not required for intron 1 site transcription arrest. (A) In vitro transcription. A 3′ deletion series was prepared, and these templates (linearized in the plasmid sequences at *Pvu*II; full length is indicated by ■) were tested for the ability to generate the intron 1 site transcripts in vitro (lanes 1 to 7) as described in Materials and Methods. The intron 1 site transcripts are indicated with an arrow. RNA marker sizes are shown at the left. In the diagram, solid lines represent ADA sequences and dashed lines indicate plasmid sequences. The full-length runoff transcript varies from 300 to 435 nt in length in part because of differences in the amount of ADA sequences as well as differences in the amount of plasmid polylinker sequences. The appropriate full-length runoff transcripts are indicated. (B) RNase protection. Unlabeled RNA from a transcription reaction was hybridized to a uniformly labeled RNA probe (lane 2) complementary to +3 to +168. Unhybridized RNA was digested with RNase, and protected products were extracted, precipitated, and electrophoresed on a 6% polyacrylamide–7 M urea gel. The expected full-length protected product corresponds to the transcribed ADA sequences present in the construct. Lanes: 1, markers (pBR322 *Msp*I); 2, undigested RNA probe (longer as a result of the inclusion of polylinker sequences); 3 to 7, protected products after hybridization of the RNA probe with yeast RNA (lane 3) or 3′ deletion constructs (lanes 4 to 7). Abbreviations: D, *Dde*I; N, *Nco*I; P, *Pvu*II.

incubation with a purified fraction of HeLa SII resulted in near-complete extension of the transcripts which had accumulated at the intron 1 pause site with a corresponding increase in the full-length runoff (Fig. 3A). Figure 3B shows a time course of a similar experiment following the addition of recombinant SII. The use of recombinant SII addresses the possibility that proteins, other than SII, present in the SII fraction purified from HeLa nuclear extracts were required for the stimulation of elongation. However, as shown in Fig. 3B, virtually all transcription complexes paused at the intron 1 site were elongated to full length within 10 min following the addition of recombinant SII. These results indicate that in the presence of NTPs, SII alone is capable of efficiently promoting the elongation of transcription complexes arrested at the intron 1 pause site.

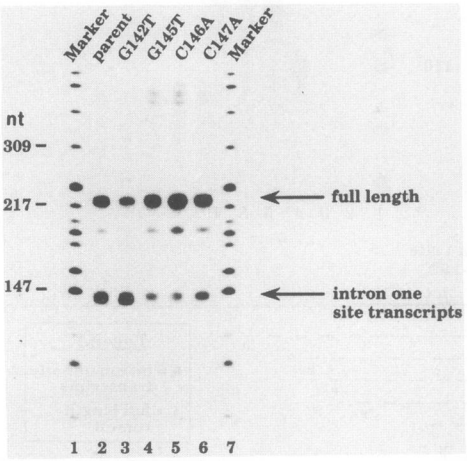
Sequences upstream of +117 are not required for the intron 1 transcription pause. To identify the template requirements for the intron 1 transcription pause site, a number of replacement and deletion constructs were prepared. When the 5′ untranslated region (+1 to +92) was either increased by 8 nt (5′UT+8), inverted (5′UTrev), or replaced by pTZ19R vector DNA (5′UTpTZ), the intron 1 site transcripts were still formed (Fig. 4, lanes 3 to 5). This finding indicated that the orientation as well as the sequence of the 5′ untranslated region are not required for the transcription arrest process. Transcription templates having internal deletions 81/98, 101/111, and 101/117 were all able to generate intron 1-arrested transcripts, shortened appropriately by the size of the deletion (Fig. 4, lanes 6 to 8). Differences in the ratio of arrested to full-length transcripts among the different mutant templates are not considered to be experimentally significant. Taken together, these results indicate that sequences upstream of +117 are not needed for the transcription pause.

Sequences downstream of +158 are not required for intron 1 transcription pausing. To define the 3′ border of the sequences necessary for intron 1 transcription pausing, a series of 3′ deletion constructs was made. When the 3′ border of the ADA sequences was +158 or longer, the intron 1-arrested transcripts accumulated (Fig. 5A, lanes 5 to 7). No intron 1-arrested transcripts were seen when the 3′ border of the ADA sequences was less than +138 (Fig. 5A, lanes 1 to 4). RNase protection analysis using labeled RNA from the in vitro transcription reactions and a ^{32}P-labeled RNA complementary to +3 to +168 of ADA revealed the full-length transcript in all cases examined (Fig. 5B, lanes 4 to 7). Intron 1-arrested transcripts were present in the

A.



B.



C.

	SEQUENCE	% Arrest	Relative Efficiency
	+140 +149		
0.4ADA	C C G T T G C C A G	61	100
G142T	C C T T T G C C A G	73	120
G145T	C C G T T T C C A G	38	62
C146A	C C G T T G A C A G	27	44
C147A	C C G T T G C A A G	44	72

FIG. 6. Effects of point mutations within the sequence determinants on the efficiency of transcription arrest. (A) Schematic of the 5′ end of the murine ADA gene. The sequences from +123 to +158 are detailed. (B) In vitro transcription. A series of point mutant constructs was prepared by recombinant PCR, and these templates (linearized in the plasmid sequences at *Hin*dIII; full length is 227 nt and indicated with an arrow) were tested for the ability to generate the intron 1 site transcripts in vitro (lanes 2 to 6). The intron 1 site transcripts are indicated with an arrow. Lanes 1, DNA markers (pBR322 *Msp*I), 2, parental template (0.4ADA); 3, G142T; 4, G145T; 5, C146A; 6, C147A. In mutant G142T, for example, the G at +142 was changed to a T. (C) Sequences of the parental template from +140 to +149 and of the four mutant templates, with the changes highlighted. Also shown are the percent arrest and relative transcription arrest for each mutant as determined following Betagen radioimage analysis.

protection assay only when transcription templates which contained sequences downstream of +158 were used (Fig. 5B, lanes 6 and 7). Hybridization to yeast RNA alone resulted in no protected products, indicating that the protected products were ADA specific (Fig. 5B, lane 3). Thus, the sequences downstream of +158 are not needed for the formation of the intron 1 site transcripts. Taken together with the previous results, these results indicate that the sequence determinants for the intron 1 pause site are located within +117 to +158.

Point mutations around the pause site influence pausing efficiency. Several point mutations were made within the sequence determinants for the intron 1 transcription pause site. In one case (G142T), the relative pausing efficiency actually increased in comparison with the parental template (Fig. 6B, lanes 2 and 3; Fig. 6C). The other three point mutations led to a decrease in the arrest efficiency (Fig. 6B, lanes 4 to 6; Fig. 6C). In the case of C146A, the efficiency of transcription arrest was decreased by more than twofold. Thus, single-point mutations within the sequence determinants can either increase or decrease the relative arrest efficiency.

The sequence determinants for intron 1 transcription pause function when placed 104 bp downstream of their natural position. To determine whether the intron 1 transcription pause site can function away from its natural position, a series of insertion templates was made by introducing heterologous DNA upstream of the pause site as diagrammed in Fig. 7. When either 4, 35, 52, or 104 bp of DNA was inserted at +92 and the resulting mutants were tested in the in vitro transcription assay, the intron 1 site transcripts were formed in each case (Fig. 7A). Differences in the ratio of arrested to full-length transcripts between the templates are not considered to be experimentally significant. To confirm that these bands represented ADA sequences, RNase protection analysis was performed. Because of the lack of an appropriate restriction site near the ADA start site, a PCR-based strategy was developed to obtain RNA probe templates as described in Materials and Methods. Protected products represent ADA-specific transcripts. As can be seen in Fig. 7B, in each case the expected intron 1-arrested transcripts were present. Therefore, the sequence determinants for the intron 1 site transcripts can be moved at least 104 bp downstream of their natural position and still function efficiently.

The intron 1 transcription pause site functions only in the native orientation. To address whether the intron 1 site sequence determinants can function in the opposite orientation in the context of ADA, the sequences from +123 to +158 were inserted in either orientation at +92 (upstream of their natural position), leaving the original intron one arrest sequences intact. When circular templates were tested in the in vitro transcription assay, the parental intron 1 site transcripts (due to the original ADA sequences) were seen in all cases (Fig. 8). With use of construct ADA + forward, a band corresponding to the intron 1 site transcripts due to the inserted sequences can be seen (Fig. 8, lane 3). It is noteworthy that some complexes which read through the first pause site were still able to pause at the second pause site. With construct ADA + reverse, no corresponding band was seen (yet the original intron 1 arrest site still functions), indicating that these sequences cannot function to create the paused transcription complexes when placed in the reverse orientation. Thus, the sequences from +123 to +158 can function to generate the intron 1-paused transcripts only in the native orientation.

The sequence determinants for intron 1-arrested transcripts can function downstream of a heterologous promoter. The last question that we wanted to address was whether the sequence determinants for the intron 1 transcription pause site can function downstream of a heterologous promoter. We placed the ADA sequences from +123 to +158 in either orientation downstream of the adenovirus major late promoter and tested the constructs in vitro. As can be seen in Fig. 9A (lanes 3 and 4), the intron one site transcripts were formed when the ADA intron 1 insert was placed downstream of the adenovirus major late promoter. Again, arrest occurs in the forward orientation only. The transcripts seen

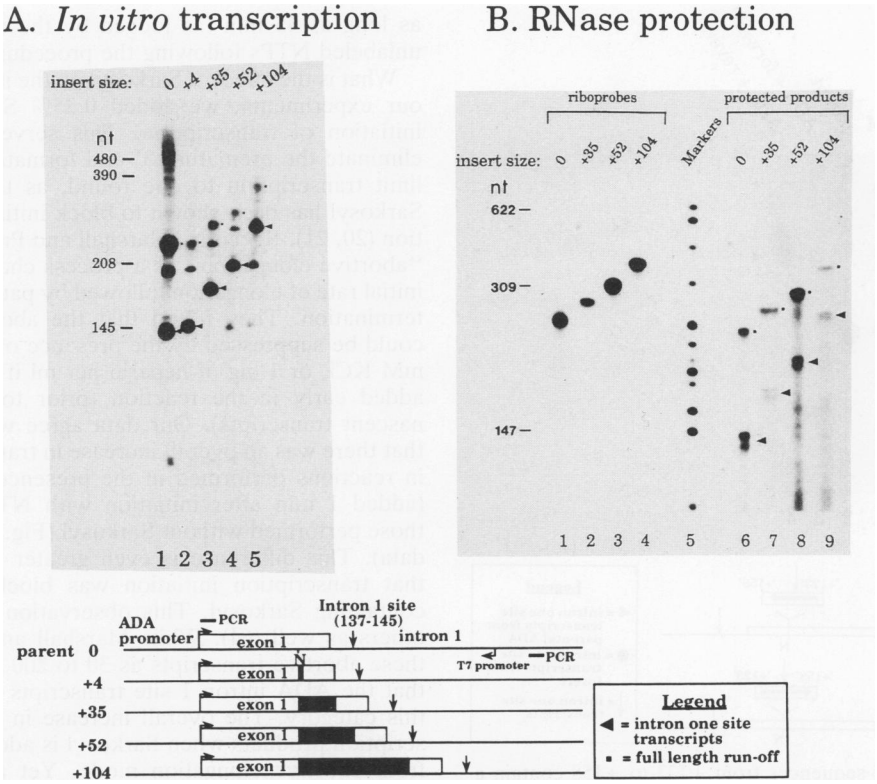


FIG. 7. Ability of the sequence determinants for the intron 1 transcription arrest site to function when placed 104 bp downstream of their natural position. A series of insertion constructs was made by inserting 4, 35, 52, or 104 bp of DNA at the unique *Nco*I site upstream of the intron 1 site (as diagrammed). (A) In vitro transcription. These constructs (linearized at a *Hin*dIII site at +227 of the 0.4ADA construct) were tested in vitro as described for the ability to form the intron 1 site transcripts. Lanes: 1, no insert; 2, 4-bp insert; 3, 35-bp insert; 4, 52-bp insert; 5, 104-bp insert. RNA size markers are indicated at the left. (B) RNase protection. Template-specific RNA probes were prepared by PCR using the primers shown. These PCR products contained ADA sequences from +3 to +206 (with the appropriate insertion as well), polylinker sequences, and a T7 promoter. RNA probes were prepared with T7 RNA polymerase (lanes 1 to 4), gel purified, and hybridized to RNA from a transcription reaction with the corresponding template. Unhybridized RNA was digested with RNase, and the protected products were extracted, precipitated, and electrophoresed on a 6% polyacrylamide–7 M urea gel. The products are shown in lanes 6 to 9. Lane 5 contains DNA markers (pBR322 *Msp*I).

corresponded to arrest within the inserted ADA sequences near the intron 1 transcription arrest site. To determine whether the appropriate start site was utilized, primer extension was performed with a ^{32}P-labeled primer complementary to the adenovirus major late promoter transcript (+31 to +49) and unlabeled RNA from a transcription reaction. Figure 9B shows that in all cases, the expected start site was utilized. The results of this experiment showed that the sequences from +123 to +158 of ADA were able to generate the intron 1-paused transcripts when placed downstream of a heterologous promoter, in an orientation-dependent fashion.

DISCUSSION

For this study, we used an in vitro transcription system to characterize the intron 1 transcription arrest site of the murine ADA gene. Using gel filtration of transcription complexes, we found that stable paused complexes can be elongated with the transcription elongation factor SII. We have also shown that a 37-bp fragment (Fig. 6A) encompassing this site is sufficient to function as a transcription arrest site downstream of the ADA and adenovirus major late promoters. Single-point mutations in the transcription pause region resulted in significant increases or decreases (by as much as 60%) in the relative pausing efficiency. We determined that these template sequences function in an orientation-dependent manner and that they function when placed an additional 104 bp downstream of their natural position.

Our previous efforts have focused on the transcription arrest site located in the first exon of the murine (26, 45) and human (6, 7) ADA genes. In those studies, both the *Xenopus* oocyte system and an in vitro transcription system using HeLa nuclear extracts were used. With both systems, using circular templates, the exon 1 arrest is prominent. However, the development of the in vitro system allowed us to examine the effect of template topology and revealed a prominent arrest site within the first intron (Fig. 1A, lane 4) (26). Thus, one difference between the exon 1 and intron 1 arrest sites is related to template topology: exon 1 transcription arrest is more efficient on circular templates than on linear templates, whereas intron 1 transcription arrest does not show a strong preference. The addition of Sarkosyl (0.25%) to the in vitro reaction to limit the reaction to only those polymerases which initiate transcription during the first minute of the reaction (also see below) resulted in the disappearance of the exon 1 site transcripts and prominent arrest at the intron 1 site regardless of template topology (compare lanes 3 and 5 in Fig. 1A) (26). The intron 1 arrest is formed by a process which is insensitive to this concen-

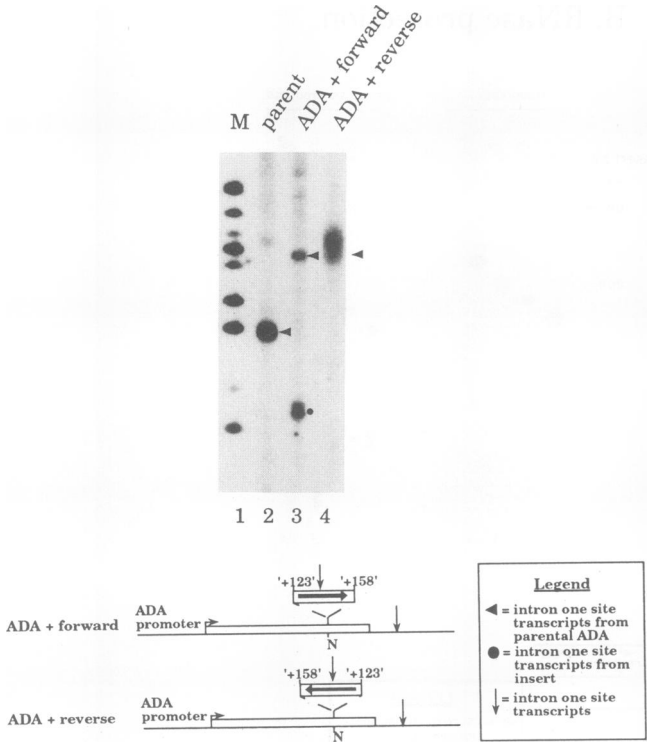


FIG. 8. Evidence that sequences from +123 to +158 contain a transcription arrest site that functions in an orientation-dependent manner. Templates containing the sequences from +123 to +158 placed in either orientation upstream (at +92) of the natural intron 1 site were prepared (diagrammed at the bottom). Circular templates were tested in vitro as described in Materials and Methods. Lanes: 1, markers (pBR322 *Msp*I); 2, parental ADA template; 3, ADA + forward (one copy of +123 to +158 in the forward orientation); 4, ADA + reverse (one copy of +123 to +158 in the opposite orientation). Abbreviation: N, *Nco*I.

tration of Sarkosyl, revealing a second difference between the exon and intron 1 arrest sites (26). These sites have no significant sequence homology to each other as well. Other genes, such as c-*myc* and c-*fos*, have multiple arrest sites at the 5′ end of the gene, as discussed in the introduction. Interestingly, in the case of c-*myc*, when the sequence determinants were placed twice as far from their natural position (310 bp increased to 600 bp), they were unable to function as efficiently (wild-type termination = 17%, reduced to <4% as distance increased [52]). We saw no such decrease in the efficiency of arrest as the distance between the start site and arrest site increased by 104 bp to 250 bp (Fig. 7). It is possible that their effect may have more to do with absolute distances, and this is a testable hypothesis.

Gel filtration experiments revealed that the ADA intron 1 arrest site is a stable RNA polymerase II pause site. We have also found that both a purified fraction of HeLa SII and recombinant SII are capable of promoting elongation of complexes through this pause site (Fig. 3). In the case of ADA, the intron 1 site transcripts were found in the column fractions containing active transcription complexes and not with the free, terminated, or runoff RNA (Fig. 2). A 10-min chase with unlabeled NTPs resulted in the appearance of full-length runoff transcripts. It is striking that the arrested complexes are quite stable during the filtration procedure and that they are able to remain in an active conformation for as long as 45 min (as judged by the ability to chase with unlabeled NTPs following the procedure).

What is the effect of Sarkosyl on the intron 1 arrest site? In our experiments, we added 0.25% Sarkosyl 1 min after initiation of transcription. This serves two purposes: to eliminate the premature 3′ end formation in exon 1 and to limit transcription to one round, as this concentration of Sarkosyl has been shown to block initiation but not elongation (20, 21). Recently, Marshall and Price (40) have defined "abortive elongation" as a process characterized by a high initial rate of elongation followed by pausing and subsequent termination. They found that the abortive characteristics could be suppressed by the presence of 0.3% Sarkosyl, 250 mM KCl, or 1 mg of heparin per ml if these reagents were added early in the reaction (prior to the release of the nascent transcripts). Our data agree with their findings in that there was an overall increase in transcription elongation in reactions performed in the presence of 0.25% Sarkosyl (added 1 min after initiation with NTPs) compared with those performed without Sarkosyl (Fig. 1A and unpublished data). This difference is even greater when one considers that transcription initiation was blocked in the reaction containing Sarkosyl. This observation has been noted by others as well (64). While Marshall and Price (40) defined these abortive transcripts as 30 to 200 nt in length, we feel that the ADA intron 1 site transcripts are not members of this category. The overall increase in the amount of transcription products when Sarkosyl is added implies a release from abortive elongation mode. Yet a 45-min incubation following the addition of Sarkosyl does not change the proportion of transcription complexes that pause at the intron 1 site (unpublished data). If the intron 1 arrest site were the result of abortive elongation, one would expect that upon addition of Sarkosyl, the appearance of these transcripts would decrease along with an increase in the amount of runoff. Instead, both transcripts increase proportionally.

Four potential mechanisms of antitermination/termination have been defined during the study of transcription arrest sites in prokaryotes and eukaryotes. First, the transcription complex may be modified by a protein (e.g., phage lambda N or Q protein [17]) which influences the ability of the polymerase to terminate at rho-independent or rho-dependent terminators. Second, the RNA transcript may form a termination secondary structure as in the bacterial attenuators (44, 55, 58, 65). In the case of premature transcription arrest in some eukaryotic and viral genes (16, 39), the sites may contain sequences which can form GC-rich stem loops followed by a stretch of uridines. In the case of ADA, this motif cannot be found within the sequence determinants identified (Fig. 6A). In our case, while the importance of an RNA secondary structure in the intron 1 arrest site has not been rigorously ruled out, for reasons stated below we believe that it is unlikely. The third type of mechanism involves DNA bending. In this case, the polymerase complex is unable to traverse a region of the DNA. This is thought to be the case in the histone H3.3 gene (49). In the fourth type of mechanism, a protein may physically block polymerase progress, as has been shown for the *lac* repressor (57), rRNA genes (9, 24), and possibly for the adenovirus major late promoter CCAAT-binding protein (10, 11). Because of preliminary data indicating specific protein binding near the intron 1 transcription pause site and because mutations near the arrest site resulted in a reduction of transcription arrest, we favor a model in which proteins bound at this site play a role in the formation of paused complexes.

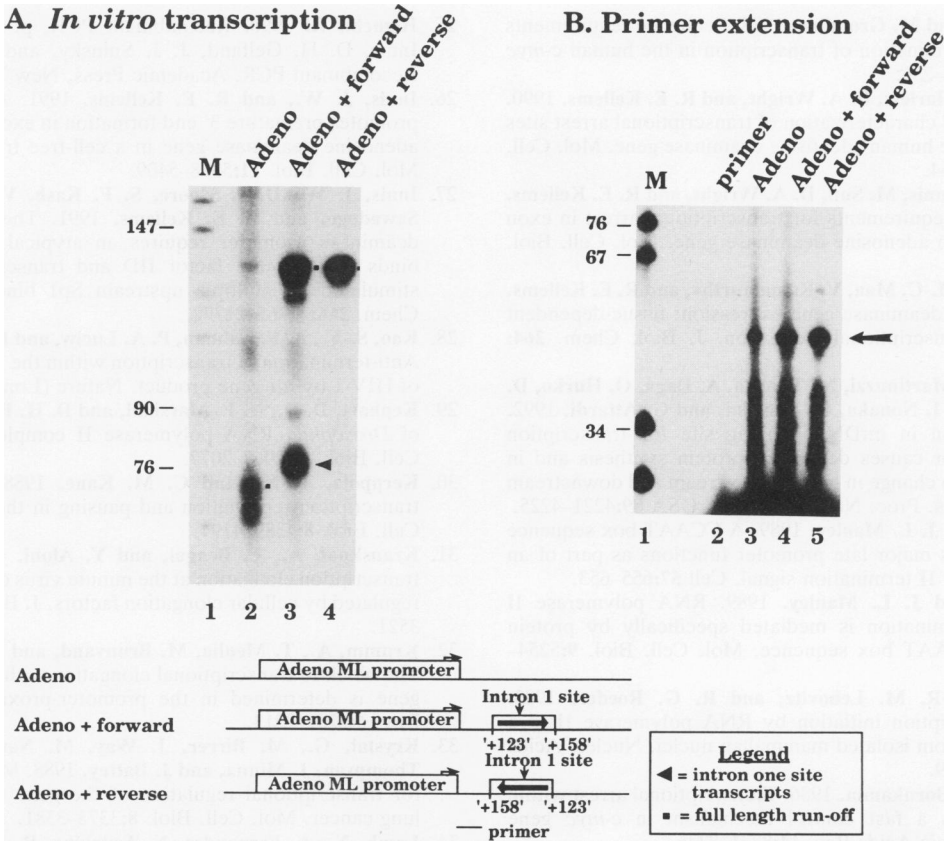


FIG. 9. Evidence that sequences from +123 to +158 arrest transcription in an orientation-dependent manner downstream of the adenovirus major late promoter. Constructs containing the ADA sequences from +123 to +158 placed in either orientation downstream of the adenovirus major late (Adeno ML) promoter were prepared (diagrammed at the bottom). (A) In vitro transcription. Templates linearized at the downstream *Sac*I site were tested in vitro as described in Materials and Methods. Lanes: 1, markers (pBR322 *Msp*I); 2, Adeno (no ADA insert; full length = 75 nt); 3, Adeno + forward (+123 to +158 in the forward orientation; full length = 128 nt); 3, Adeno + reverse (+123 to +158 in the opposite orientation; full length = 128 nt). (B) Primer extension for mapping of transcription initiation sites. A ^{32}P-end-labeled primer complementary to +31 to +49 of the adenovirus transcript was hybridized to unlabeled RNA from a transcription reaction with the three different templates. The primer was extended and products were analyzed on an 8% polyacrylamide–7 M urea gel. Lanes: 1, markers (pBR322 *Msp*I); 2, primer; 3 to 5, extended products from reactions with RNA from Adeno (lane 3), Adeno + forward (lane 4), or Adeno + reverse (lane 5). Extended products are 49 bp in length and indicated with an arrow.

Factors identified which affect transcription elongation include SII (3, 46, 48, 56), TFIIF (14), TFIIX (47), and the recently defined P-TEF and N-TEF (29, 40). SII is able to promote read-through of the histone H3.3 (59) and adenovirus major late promoter (46, 48) arrest sites in a time-dependent manner. TFIIX was originally identified as a factor involved in stimulating transcription from the adenovirus major late promoter when sequences downstream of +33 relative to the cap site were present (47). TFIIF and TFIIX as well as SII are able to relieve the elongation block in the minute virus of mice (3, 31). We have found that both a purified fraction of HeLa SII and recombinant SII are capable of promoting read-through of the ADA intron 1 pause site (Fig. 3). The effects of the other factors on the ADA intron 1 pause site is unknown. It will be of interest to determine whether other elongation factors promote read-through of the ADA intron 1 pause site. Also, it remains to be seen whether this pause site functions to block RNA polymerase I or III transcription. If so, it will be important to determine whether SII is able to promote read-through at this site with these polymerases. These hypotheses are currently being examined.

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