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Sequence Requirements for Transcriptional Arrest in Exon 1 of the Human Adenosine Deaminase Gene

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We have previously demonstrated that a transcriptional arrest site exists in exon 1 of the human adenosine deaminase (ADA) gene and that this site may play a role in ADA gene expression (Z. Chen, M. L. Harless, D. A. Wright, and R. E. Kellems, Mol. Cell. Biol. 10:4555–4564, 1990). Sequences involved in this process are not known precisely. To further define the template requirements for transcriptional arrest within exon 1 of the human ADA gene, various ADA templates were constructed and their abilities to confer transcriptional arrest were determined following injection into *Xenopus* oocytes. The exon 1 transcriptional arrest signal functioned downstream of several RNA polymerase II promoters and an RNA polymerase III promoter, implying that the transcriptional arrest site in exon 1 of the ADA gene is promoter independent. We identified a 43-bp DNA fragment which functions as a transcriptional arrest signal. Additional studies showed that the transcriptional arrest site functioned only in the naturally occurring orientation. Therefore, we have identified a 43-bp DNA fragment which functions as a transcriptional arrest signal in an orientation-dependent and promoter-independent manner. On the basis of our findings, we hypothesize that tissue-specific expression of the ADA gene is governed by factors that function as antiterminators to promote transcriptional readthrough of the exon 1 transcriptional arrest site.

Recently emerging evidence suggests that transcriptional termination and antitermination mechanisms play an important role in the regulation of gene expression (for a review, see reference 51). This type of control can occur very early after transcription initiation and, in some cases, has been documented to be under metabolic or developmental control (4, 6, 13, 47). Premature termination of transcription has been found in many cellular genes, such as c-*myc* (6, 16, 26, 39), L-*myc* (28), c-*fos* (18, 30), c-*myb* (4, 53), the human histone gene *H3-3* (44), the adenosine deaminase (ADA) gene (8, 9, 31, 33, 41), the human epidermal growth factor receptor gene (19), the *Drosophila hsp-70* gene (47), and several other *Drosophila* genes (48). In addition, control of transcription elongation is associated with the regulated expression of several viral transcription units, including those of simian virus 40 (20), adenovirus type 2 (34, 38), polyomavirus (50), minute virus of mice (3, 45), and human immunodeficiency virus (HIV) (25). In some cases, DNA sequences responsible for premature termination of transcription have been narrowed down to a relatively short DNA fragment. Several signals involving antitermination have also been determined (7, 36, 37, 42).

In the murine c-*myc* gene, a 180-bp DNA fragment, including the 3′ half of exon 1 (37), is sufficient to mediate a block to transcription within the intron of a heterologous gene (54). In the human c-*myc* gene, a 95-bp region from exon 1 is sufficient to confer transcriptional arrest when linked downstream of some heterologous promoters, as demonstrated by analysis of c-*myc* transcripts after injection of transcription templates into *Xenopus* oocytes (7). In both murine and human c-*myc* genes, termination occurs at thymidine-rich sequences and is preceded by RNA sequences with the potential to form a stem-loop structure. However, deletion analysis indicates that the T residues are not necessary to specify premature termination and that a region upstream with dyad symmetry is required to confer termination (7). An element of the HIV type 1 (HIV-1) long terminal repeat called IST (inducer of short transcripts) has been identified and it is responsible for the production of short prematurely terminated transcripts (42). The IST element is located between −3 and +82 relative to the cap site and is capable of activating the synthesis of short transcripts from several RNA polymerase II promoters and an RNA polymerase III promoter. Because IST overlaps with the TAR (*trans*-activation response) sequence (24, 49), the exact sequences required for IST function remain to be determined. It appears that IST functions by inducing the formation of transcription complexes that are not efficient in transcription elongation. However, in the presence of the HIV transactivator Tat protein, modified transcription complexes which elongate efficiently to make full-length RNA are formed (42). By complementing Tat-defective viruses with the Tat protein, it has been shown that transactivation results primarily from an increase in transcription elongation, suggesting that Tat may be a gene-specific transcription elongation factor (17). In the murine c-*fos* gene, a 103-bp intron 1 fragment is sufficient to block transcription in a cell-free transcription system (36). Although a stable hairpin structure can be deduced from the nucleotide sequences preceding the termination site, its importance remains to be determined. Interestingly, in murine macrophages, the intron 1 transcription block of the c-*fos* gene is modulated by a calcium response element (13). The induction of c-*fos* transcription by a number of agents is accompanied by a release of the elongation block; however, this effect is prevented in the absence of calcium, suggesting a requirement for intracellular calcium to achieve unhindered tran-

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scription throughout the c-*fos* gene. Despite recent progress, a common consensus has yet to be found among all the sites of premature transcription termination and the molecular mechanism of transcriptional arrest is not understood.

Previously, we have provided evidence that a transcriptional arrest site exists at the 5′ end of murine and human ADA genes and that this arrest site is involved in the regulation of ADA gene expression (8, 9, 33, 41). Briefly, in tissues or cells with a high level of ADA gene expression, transcriptional activity can be detected across the entire gene. On the other hand, transcriptional activity can be detected only in the 5′ end of the gene in tissues or cells with a low level of ADA gene expression. Transcriptional analysis of the human ADA gene in *Xenopus* oocytes indicates that abundant short ADA transcripts accumulate in the injected oocytes. The 3′ end of the short transcript maps within exon 1 and corresponds to the region of transcriptional arrest detected in nuclear run-on experiments. Thus, regulation of transcription elongation by modulating premature termination within exon 1 of the ADA gene may play a major role in ADA gene expression. To further delineate the *cis*-acting element(s) responsible for transcriptional arrest in the human ADA gene and to investigate the molecular mechanism of the arrest process, we constructed various ADA templates and analyzed their abilities to confer transcriptional arrest in the *Xenopus* oocyte transcription system. We have identified a 43-bp DNA fragment of the human ADA gene which contains a transcriptional arrest signal. This signal is orientation dependent and functions downstream of several heterologous promoters, including an RNA polymerase III promoter, indicating that it functions autonomously as a transcriptional arrest signal. The role of the transcriptional arrest signal in the regulation of ADA gene expression is discussed.

MATERIALS AND METHODS

Plasmid construction. Construction of EP0.4 has been described previously (8). EP0.4d was constructed by digesting EP0.4 with *Nco*I and *Bss*HII, filling in the ends by Klenow polymerase, and recircularizing in the absence of the 43-bp *Nco*I-*Bss*HII fragment. EP0.4i was constructed in a similar way except that recircularization was performed in the presence of the *Nco*I-*Bss*HII fragment. For EP0.4r, the *Nco*I-*Bss*HII fragment was filled in and ligated to EP0.4d in which the *Nar*I site had been cleaved and filled in by Klenow polymerase treatment. To construct promoter-switch plasmids, a promoterless ADA plasmid, SP0.24, was made by ligating the 246-bp *Sac*II-*Pst*I fragment of the human ADA gene (bp 3932 to 4177) into the *Sac*II-*Pst*I double-digested Bluescript vector KS(+). TK-ADA was constructed by ligating a 203-bp *Pvu*II-*Mnl*I fragment containing the herpesvirus thymidine kinase (TK) gene promoter to SP0.24 which had been cleaved at the *Sac*II site and repaired by T4 DNA polymerase. CMV-ADA was constructed in a similar fashion except that a 806-bp *Eco*RI-*Sau*3AI DNA fragment containing the cytomegalovirus (CMV) immediate early promoter was filled in by Klenow polymerase and used in the ligation. To make MLP-ADA, a 293-bp *Xho*I-*Pvu*II fragment containing the adenovirus major late promoter (MLP) was subcloned into the *Xho*I-*Sma*I double-digested Bluescript vector KS(+) to derive MLP-KS. To construct MLP-ADA4 and MLP-ADA14, *Xba*I-digested MLP-KS was deleted towards the transcription start site by S1 nuclease, followed by *Xho*I digestion, Klenow polymerase filling in, and purification of a pool of small MLP fragments different in length. The DNA fragments were ligated to *Sac*II-digested, T4 DNA polymerase-repaired SP0.24. To construct MLP-ADA57, MLP-KS was cleaved at the *Xba*I site in the polylinker and ligated to the 246-bp *Sac*II-*Pst*I fragment of the human ADA gene (bp 3932 to 4177) which had been treated with T4 DNA polymerase and Klenow polymerase. Construct mU6-ADA was made by subcloning the T4 DNA polymerase and Klenow polymerase-treated *Sac*II-*Pst*I fragment of the ADA gene into mU6(−315/1) (43), which was linearized at the *Eco*RI site and filled in by Klenow polymerase. Templates TERc and TERr were constructed as follows. The Klenow polymerase-treated 43-bp *Nco*I-*Bss*HII fragment was inserted into the Klenow polymerase-treated *Xba*I site of MLP-KS in either the correct (TERc) or reverse (TERr) orientation. The authentic structures of all constructs have been confirmed by restriction endonuclease digestions and dideoxy chain termination sequence analysis (52).

Oocyte injection and RNA isolation. The injection of supercoiled plasmid DNA into *Xenopus* oocytes and the isolation of RNA have been described previously (8). Typically, 4 ng of DNA was injected into each germinal vesicle, except that in the case of mU6-ADA, 0.5 ng of plasmid DNA was injected per oocyte. In coinjection experiments, EP0.4 and mU6 were mixed at a ratio of 7:1 (wt/wt) and 4 ng of mixed DNA was injected into each oocyte. For α-amanitin controls, plasmid DNA was resuspended in 10 μg of α-amanitin per ml before injection. A pool of 8 to 10 oocytes per sample was collected 24 h after injection, and the RNA was isolated.

Northern blot analysis of RNA. Two micrograms of oocyte RNA per sample was separated by electrophoresis through 6% polyacrylamide–8 M urea denaturing gels and electroblotted onto nylon membranes (Zeta-Probe). ADA-specific transcripts were detected with a ^{32}P-labeled oligonucleotide complementary to the ADA mRNA from +13 to +37 (8). Another oligonucleotide, 5′ GAACTAGTGGATCCCCC CTG 3′, was used to detect transcripts generated from templates TERc and TERr. Hybridization as well as washing conditions have been described previously (8).

Primer extension analysis. The same primers utilized in Northern blot analysis were employed as primers for primer extension analysis, and 2 μg of oocyte RNA was used in each assay. The procedure has been described before (8).

Ribonuclease protection assay. ^{32}P-labeled RNA probes were synthesized by T7 RNA polymerase from *Sma*I-digested EN0.3 (8). Two micrograms of oocyte RNA was mixed with RNA probe (3×10^5 cpm), precipitated, resuspended in 20 μl of hybridization buffer [40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.4), 80% formamide, 300 mM NaCl, 5 mM EDTA], and hybridized at 47°C for 5 h. Ribonuclease digestion was performed at 37°C for 1 h by adding 0.35 ml of digestion buffer containing 10 mM Tris (pH 7.5), 300 mM NaCl, 5 mM EDTA, and 30 μg of ribonuclease A per ml as well as 1.9 U of RNase T_1 per ml (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). After digestion, 10 μl of 20% sodium dodecyl sulfate and 2.5 μl of a proteinase K solution (20 mg/ml) (Boehringer Mannheim Biochemicals) were added and the mixtures were incubated for 15 min at 37°C. RNA was phenol extracted, ethanol precipitated, and separated by electrophoresis through 6% polyacrylamide–8 M urea gels.

RESULTS

Short ADA-specific transcripts are generated from ADA templates injected into *Xenopus* oocytes. We have shown previously that the *Xenopus* oocyte transcription system is

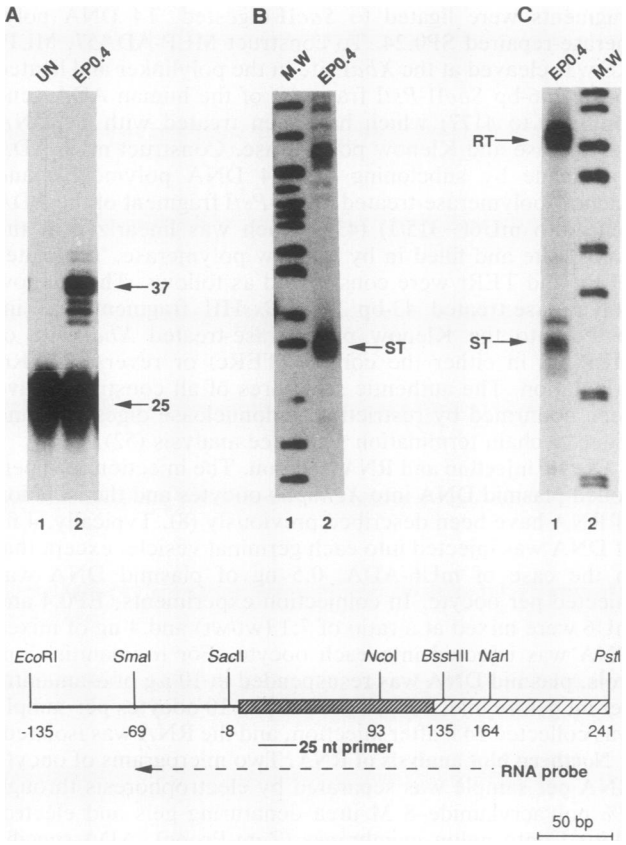

FIG. 1. Analysis of transcripts generated from ADA templates injected into *Xenopus* oocytes. ADA template EP0.4, shown at bottom, was injected into oocytes, and the RNA generated from this template was isolated and characterized. Exon 1 extends from position 1 to 128. (A) Primer extension analysis of transcription initiation site. A ^{32}P-labeled, ADA-specific 25-nucleotide (nt) oligonucleotide (+13 to +37) was hybridized to 2 μg of RNA and extended by avian myeloblastosis virus reverse transcriptase, and the extended products were separated on a 10% sequencing gel. The approximate sizes (in nucleotides) of the primer and the extended products are indicated. Lanes: 1, uninjected control; 2, EP0.4 injection. (B) Northern analysis. Two micrograms of oocyte RNA was separated on a 6% polyacrylamide–8 M urea gel, electroblotted onto a nylon membrane, and probed with the same 25-nucleotide oligonucleotide as in primer extension analysis. The position of the ADA-specific short transcripts (ST) is indicated. Lanes: 1, molecular weight markers (radioactively labeled pBR322-*Msp*I digest); 2, EP0.4 injection. (C) 3′ end analysis. The 3′ ends of ADA transcripts were determined by ribonuclease protection analysis. A ^{32}P-labeled RNA probe antisense to the *Sma*I-*Nar*I region of the ADA coding strand was synthesized by T7 RNA polymerase and hybridized to RNA. After ribonuclease digestion, RNA was extracted and separated on a 6% polyacrylamide–8 M urea gel. The protected readthrough transcripts (RT) and short transcripts (ST) of the ADA gene are indicated. Lanes: 1, EP0.4 injection; 2, molecular weight markers.

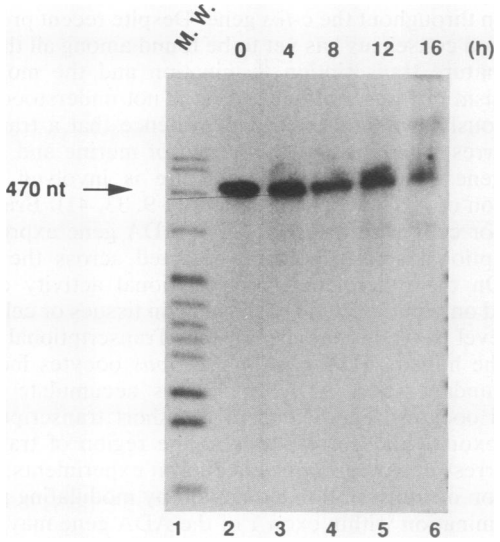

FIG. 2. An in vitro synthesized ADA transcript is not cleaved into smaller RNA in *Xenopus* oocytes. An ADA transcript was synthesized by T7 RNA polymerase in the presence of [α-^{32}P]UTP, purified, and injected into the cytoplasm of *Xenopus* oocytes (5×10^4 cpm per oocyte). After injection, oocyte RNA was collected at 4, 8, 12, and 16 h, as indicated, and separated on a 6% polyacrylamide–8 M urea gel. After electrophoresis, the gel was placed against XAR-5 film and exposed at −70°C overnight. The 470-nucleotide (nt) RNA is indicated. M.W., molecular weight markers.

able to recognize a transcriptional arrest signal in exon 1 of the human ADA gene (8). Therefore, we employed the oocyte injection system as our assay to further characterize the exon 1 transcriptional arrest signal. As shown in Fig. 1, when ADA construct EP0.4, which contains the ADA promoter, exon 1, and 114 bp of intron 1, was injected into *Xenopus* oocytes, ADA-specific transcripts were generated. 5′ end analysis of the ADA transcripts demonstrated that transcription initiated from the same position as in human cells (Fig. 1A, lane 2). Significantly, short ADA transcripts were detected by Northern blot analysis, and these short transcripts were estimated to be approximately 105 nucleotides in length (Fig. 1B, lane 2). Transcripts larger than 250 nucleotides were read-through transcripts into vector sequences (Fig. 1A, lane 2). On the basis of their size, the short ADA transcripts should have their 3′ ends inside exon 1, which is 128 bp long. To verify this, an RNase protection assay was performed to determine the 3′ end of ADA transcripts generated in oocytes. Short ADA transcripts were indeed detected, and they were approximately 105 nucleotides long (Fig. 1C, lane 1). The doublet pattern of the short transcripts may reflect 3′ end heterogeneity of the short transcripts or may be due to "nibbling" activity of the RNase under our assay conditions. Full-length transcripts were also detected, and they correspond to ADA transcripts that did not terminate in exon 1. On the basis of these and our previous results (8), we estimate that the short ADA transcripts comprise 30 to 40% of all the steady-state ADA transcripts generated in oocytes.

It is possible that the short ADA transcripts were generated by an RNA processing event that cleaves a longer ADA transcript at specific sites to generate the shorter transcripts. To test this possibility, ^{32}P-labeled, 470-nucleotide ADA transcripts were synthesized in vitro and injected into oocytes. Oocyte RNA was isolated at various times after injection and fractionated on a 6% polyacrylamide–8 M urea gel. As shown in Fig. 2, the in vitro synthesized ADA transcripts were stable in oocytes for 16 h or more after injection and were not processed to discrete-sized shorter transcripts (Fig. 2, lanes 3 to 6). This finding suggests that short ADA transcripts were not formed by a posttranscriptional processing event that specifically cleaves a larger ADA transcript. Therefore, it is likely that the 3′ ends of the

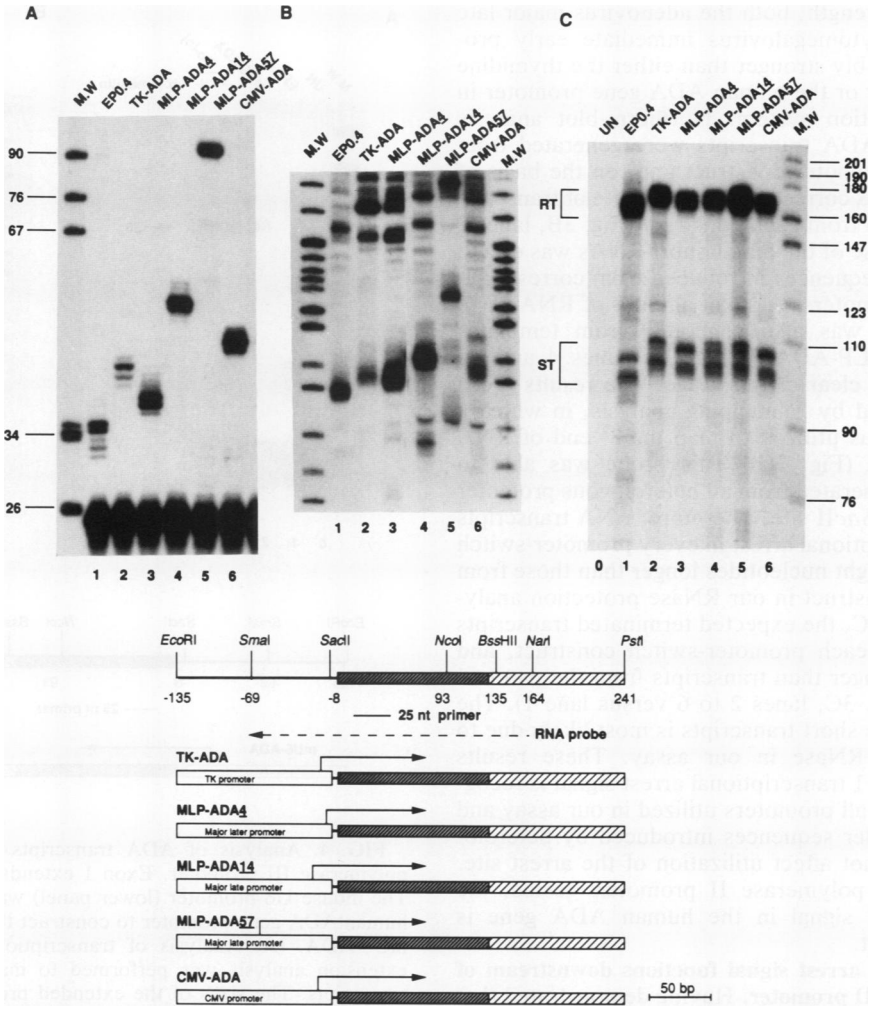

FIG. 3. Evaluation of the promoter dependence of the transcription arrest process. In the bottom panel, the ADA gene promoter element, the *Eco*RI-*Sac*II fragment, was replaced with heterologous RNA polymerase II promoters, including herpesvirus thymidine kinase gene promoter (TK-ADA), adenovirus major late promoter (MLP-ADA), and cytomegalovirus immediate early promoter (CMV-ADA). Exon 1 of the ADA gene extends from position 1 to 128. Open boxes denote heterologous promoters; ⌐→ denotes transcription initiation site. (A) Analysis of transcription initiation site. Primer extension analysis was performed by using the ADA-specific 25-nucleotide (nt) oligonucleotide. The sizes of the molecular weight markers (radioactively labeled pBR322-*Msp*I digest), given in nucleotides, are indicated. (B) Northern analysis. (C) Ribonuclease protection analysis. 3′ ends of the RNA transcripts were mapped by ribonuclease protection assays as described in Materials and Methods. The protected products were separated on a 6% polyacrylamide–8 M urea gel. Prematurely terminated short transcripts (ST) and read-through transcripts (RT) are indicated. Lanes: 1, EP0.4 injection; 2, TK-ADA injection; 3, MLP-ADA4 injection; 4, MLP-ADA14 injection; 5, MLP-ADA57 injection; 6, CMV-ADA injection.

short RNA are formed by a transcriptional arrest mechanism rather than by posttranscriptional processing. However, a cotranscriptional process cannot be ruled out at this stage. From the findings presented above, we conclude that when ADA gene fragments are injected into *Xenopus* oocytes, short ADA transcripts, approximately 105 nucleotides in length, accumulate. The 5′ ends of these transcripts map to the authentic ADA transcription initiation site, and the 3′ ends map within exon 1.

Generation of the short ADA-specific transcript is promoter independent. In order to determine whether transcriptional arrest in exon 1 of the human ADA gene is promoter dependent, a number of promoter-switch constructs were prepared. In these constructs, an *Eco*RI-*Sac*II fragment encompassing the ADA promoter was replaced by heterologous promoters, including the herpesvirus thymidine kinase gene promoter, the adenovirus major late promoter, or the cytomegalovirus immediate early promoter (Fig. 3). In every promoter-switch construct, the transcription initiation site was situated upstream of the normal transcription start site in EP0.4. In addition, we prepared three different MLP-ADA constructs which initiated transcription at 4, 14, or 57 nucleotides upstream of the normal transcription initiation site. Therefore, possible contributions of different 5′ leader sequences from these promoters to the downstream transcriptional arrest process could also be evaluated. 5′ mapping analysis of RNA transcripts demonstrated that in all of the promoter-switch constructs, transcription initiated farther upstream than in the parental construct EP0.4: 7 bp farther in TK-ADA, 11 bp farther in CMV-ADA, and 4, 14, and 57 bp farther in MLP-ADA4, MLP-ADA14, and MLP-ADA57, respectively (Fig. 3A, lanes 1 to 6). Furthermore, in

terms of promoter strength, both the adenovirus major late promoter and the cytomegalovirus immediate early promoter were considerably stronger than either the thymidine kinase gene promoter or the human ADA gene promoter in the oocyte transcription system. Northern blot analysis revealed that short ADA transcripts were generated from each heterologous promoter construct and, on the basis of their size, their 3′ ends corresponded to the site of transcriptional arrest expected from each construct (Fig. 3B, lanes 2 to 6). The size increase of the major short RNAs was due to additional 5′ leader sequences introduced from corresponding heterologous promoters. A small amount of RNA 88 to 92 nucleotides long was also generated from templates MLP-ADA14 and MLP-ADA57 (Fig. 3B, lanes 4 and 5). Their origins are not clear at this stage. The results given above were confirmed by 3′ mapping analysis, in which a single RNA probe was utilized to map the 3′ end of RNA transcripts generated (Fig. 3C). This probe was able to hybridize to RNA generated from all heterologous promoter constructs up to the *Sac*II site. Therefore, RNA transcripts generated by transcriptional arrest in every promoter-switch construct would be eight nucleotides longer than those from a wild-type ADA construct in our RNase protection analyses. As seen in Fig. 3C, the expected terminated transcripts were detected from each promoter-switch construct, and they were slightly longer than transcripts from the parental construct EP0.4 (Fig. 3C, lanes 2 to 6 versus lane 1). The doublet pattern of the short transcripts is most likely due to nibbling activity of RNase in our assay. These results suggest that the exon 1 transcriptional arrest signal is recognized downstream of all promoters utilized in our assay and that the extra 5′ leader sequences introduced by heterologous promoters did not affect utilization of the arrest site. Thus, for the RNA polymerase II promoters tested, the transcriptional arrest signal in the human ADA gene is promoter independent.

The transcriptional arrest signal functions downstream of an RNA polymerase III promoter. Having demonstrated that the transcriptional arrest signal in exon 1 of the human ADA gene can be recognized by transcription complexes initiated from a number of RNA polymerase II promoters, we wanted to determine whether the arrest signal would function in the context of an RNA polymerase III transcription unit. For this purpose, the mouse U6 promoter was chosen because it functions well in oocytes and it is transcribed by RNA polymerase III (8, 43). The new construct, mU6-ADA, was injected into oocytes either alone or in the presence of EP0.4, which served as an internal control. In mU6-ADA, the mouse U6 promoter introduced an extra 18 nucleotides of 5′ leader sequence upstream of the normal transcription initiation site utilized in EP0.4 (Fig. 4A, lanes 1 and 2). Thus, in EP0.4 and mU6-ADA coinjections, transcripts generated from mU6-ADA were 18 nucleotides longer (Fig. 4A, lane 3). Furthermore, transcripts synthesized from mU6-ADA were resistant to low concentrations of α-amanitin, while transcripts generated from EP0.4 were absent in the same concentrations of α-amanitin (Fig. 4A, lane 4), suggesting that EP0.4 was transcribed by RNA polymerase II and mU6-ADA was transcribed by RNA polymerase III. If the transcriptional arrest signal was recognized in mU6-ADA, the expected short, terminated transcript would be approximately 124 nucleotides in length. Indeed, an RNA species of about 124 nucleotides was detected following injection of mU6-ADA (Fig. 4B, lanes 2 and 3), and it was not affected by the presence of low concentrations of α-amanitin (Fig. 4B, lane 4). An extra species of RNA 147 nucleotides in length was generated from mU6-ADA (Fig. 4B, lanes 2 to 4), which may result from the presence of an RNA polymerase III-like termination signal near the exon 1-intron 1 junction of the human ADA gene. Taken together, the results given above indicate that the transcriptional arrest signal functions even when it is placed downstream of an RNA polymerase III promoter.

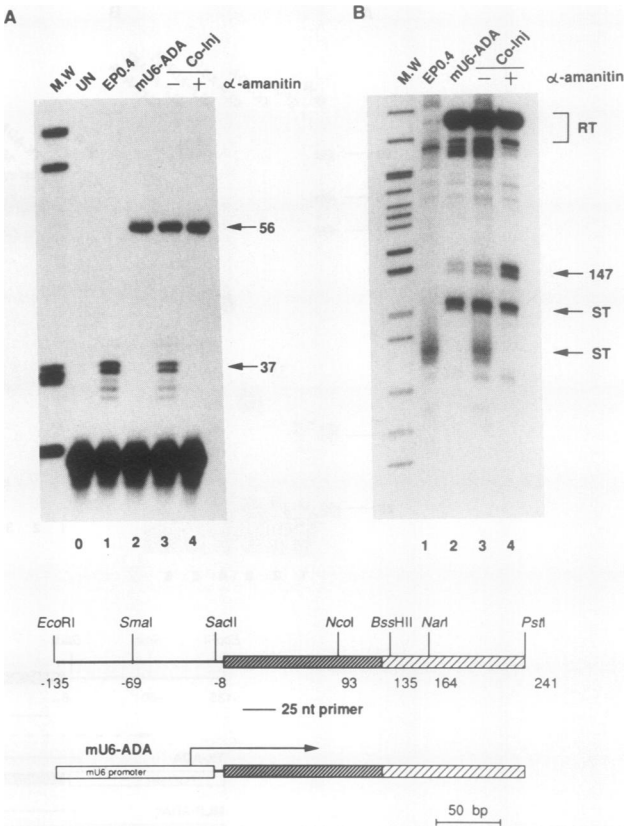

FIG. 4. Analysis of ADA transcripts initiated from an RNA polymerase III promoter. Exon 1 extends from position 1 to 128. The mouse U6 promoter (lower panel) was utilized to replace the human ADA gene promoter to construct the transcription template mU6-ADA. (A) Analysis of transcription initiation site. Primer extension analysis was performed to map the 5′ ends of RNA transcripts. The sizes of the extended products are indicated. (B) Northern analysis. The short transcripts (ST) and the readthrough transcripts (RT) are indicated. Lanes: 0, uninjected control; 1, EP0.4 injection; 2, mU6-ADA injection; 3, EP0.4 and mU6-ADA coinjection; 4, EP0.4 and mU6-ADA coinjection in the presence of 10 μg of α-amanitin per ml.

A 43-bp segment of the human ADA gene specifies transcriptional arrest in an orientation-dependent manner. To delineate the *cis*-acting elements involved in transcriptional arrest, we constructed mutant templates containing this region and tested the effect of the mutations on transcriptional arrest. First, an internal deletion template, EP0.4d, was constructed in which a 43-bp *Nco*I-*Bss*HII fragment was deleted (Fig. 5). The effect of the deletion on transcriptional arrest was determined by examining the ability of EP0.4d to generate short ADA transcripts following injection into *Xenopus* oocytes. As shown in Fig. 5, when the wild-type ADA construct EP0.4 was injected into oocytes, short ADA transcripts accumulated (Fig. 5B, lane 1). When the 43-bp *Nco*I-*Bss*HII fragment was deleted, the short transcripts

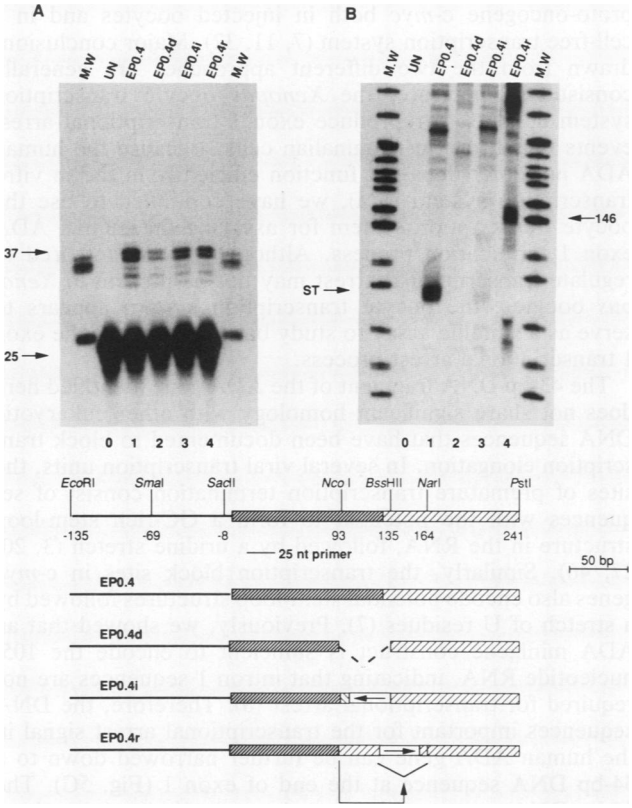

FIG. 5. Identification of a *cis*-acting element responsible for transcription arrest. In the bottom panel, RNA from uninjected oocytes or oocytes injected with ADA templates was prepared 24 h after injection and subjected to further analyses. Symbols: ▨, exon 1 sequences; ▨, intron 1 sequences; box with arrow denotes the 43-bp *Nco*I-*Bss*HII fragment, and thick line denotes 5′ flanking sequences. An arrow inside a box indicates the direction of the insert. EP0.4 contains the ADA promoter, exon 1, and 114 bp of intron 1. The 43-bp *Nco*I-*Bss*HII fragment was deleted in EP0.4d, and the same fragment was inverted in EP0.4i. EP0.4r was constructed by inserting the 43-bp DNA fragment into the *Nar*I site of EP0.4d. (A) 5′ mapping analysis. The transcription initiation site of the ADA gene was determined by primer extension analysis as described in the legend to Fig. 1. The sizes (in nucleotides) of the primer and the extended products are indicated. (B) Northern analysis. ADA-specific transcripts were detected with the same 25-nucleotide oligonucleotide as in Fig. 1. The short transcript (ST) is indicated. Lanes: 0, uninjected control; 1, EP0.4 injection; 2, EP0.4d injection; 3, EP0.4i injection; 4, EP0.4r injection. M. W., size markers (radioactively labeled pBR322-*Msp*I digest).

were no longer observed (Fig. 5B, lane 2), while transcription initiation from this construct (EP0.4d) was not significantly affected (Fig. 5A, lane 2). These results indicate that sequences within the 43-bp DNA fragment are necessary for the generation of the short transcripts. To determine whether the 43-bp DNA fragment functions in an orientation-dependent manner, it was inverted in mutant EP0.4i (Fig. 5) so that its orientation was opposite to that in wild-type construct EP0.4. It was evident that the inversion of the 43-bp DNA fragment greatly reduced the generation of the short ADA transcripts (Fig. 5B, lane 3), suggesting that the requirement for the 43-bp fragment is orientation dependent. Finally, to assess whether the 43-bp *Nco*I-*Bss*HII fragment is able to restore transcriptional arrest, it was inserted in the correct orientation into EP0.4d at the *Nar*I site 37 bp 3′ to the exon-intron boundary to make construct EP0.4r. Short ADA transcripts approximately 146 nucleotides long would be expected if the 43-bp DNA insertion is able to restore transcriptional arrest within the inserted segment downstream. As predicted, short ADA transcripts approximately 146 nucleotides in length were detected (Fig. 5B, lane 4), implying that the *Nco*I-*Bss*HII fragment is able to confer transcriptional arrest in the context of the human ADA gene when placed further downstream in intron 1. In addition, manipulations of the 43-bp DNA fragment neither affected the transcription start site nor changed the transcription efficiency in these mutants (Fig. 5A, lanes 1 to 4). Therefore, on the basis of the above findings, we conclude that a 43-bp DNA fragment encompassing the 3′ portion of exon 1 and a very small piece of intron 1 contains a transcriptional arrest signal and that this signal functions in an orientation-dependent manner.

The 43-bp DNA fragment functions autonomously to confer transcriptional arrest. Although in Fig. 5 we showed that the 43-bp *Nco*I-*Bss*HII fragment was able to restore transcriptional arrest when taken out of its usual context and placed further downstream, the DNA fragment was still in the context of the human ADA gene. It was not clear from those experiments whether it was sufficient to confer transcriptional arrest within the context of a foreign gene. To test whether the 43-bp *Nco*I-*Bss*HII fragment of the human ADA gene is able to function independently, the DNA fragment was inserted 60 bp downstream of the adenovirus major late promoter in both orientations. The derived templates, TERc (c for correct orientation) and TERr (r for reverse orientation) were injected into oocytes, and the resulting transcriptional products were analyzed. As shown by Northern analysis, the majority of transcripts generated from TERr were read-through transcripts (Fig. 6B, lane 1). On the other hand, when template TERc was injected into oocytes, two distinct classes of RNA were generated, longer transcripts which correspond to transcription readthrough of the insert and short transcripts 72 ± 5 nucleotides in length (Fig. 6B, lane 2). The 3′ ends of the latter class of transcripts were located inside the 43-bp DNA fragment and corresponded approximately to the same site where transcription prematurely terminated in the human ADA templates to produce the short transcripts. It is not clear why short transcripts generated from TERc have heterogenous 3′ ends. A very small amount of short transcripts was generated from TERr, raising the possibility that the termination signal may have a slight effect on transcriptional elongation in the opposite orientation. However, we conclude that, in the *Xenopus* oocyte transcription system, the 43-bp DNA fragment functions autonomously as a transcriptional arrest signal and this effect is largely orientation dependent.

DISCUSSION

In this study, we have shown that a 43-bp DNA fragment from the 5′ end of the human ADA gene contains a transcriptional arrest signal and that it functions in an orientation-dependent manner in the oocyte transcription system. The arrest signal functioned downstream of several heterologous promoters, including an RNA polymerase III promoter. Therefore, the transcriptional arrest signal appears to be a general signal in that it is recognized not only by RNA polymerase II but also by RNA polymerase III. Although our studies were based on analyses of ADA transcripts generated from ADA templates injected into *Xenopus* oocytes, we believe, as do others (7), that this approach is

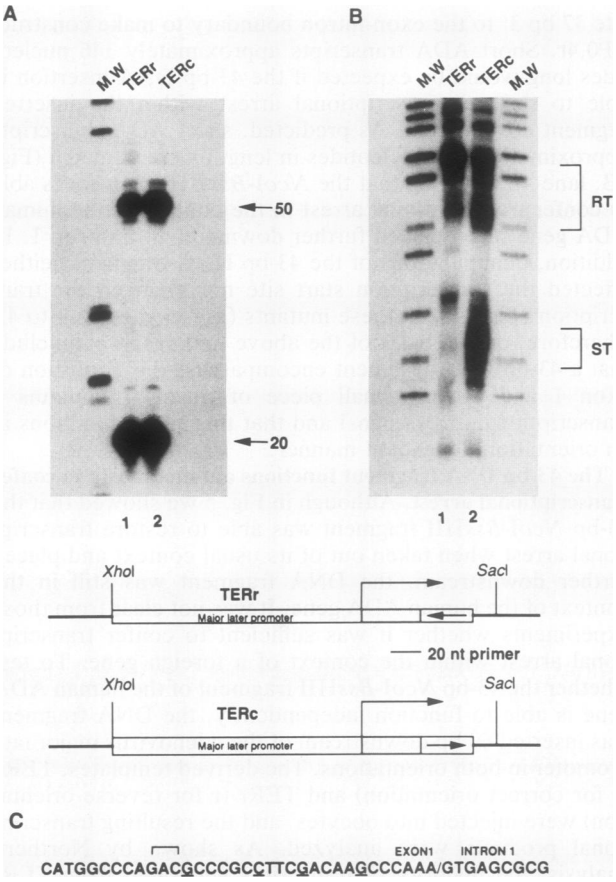

FIG. 6. Determination of the ability of the 43-bp *Nco*I-*Bss*HII fragment to confer transcription arrest. As shown below the gels, the 43-bp *Nco*I-*Bss*HII fragment was inserted downstream of the adenovirus major late promoter either in the correct orientation (TERc) or in the reverse orientation (TERr). Thick line denotes vector sequences, open box denotes the major late transcription unit of adenovirus, box with arrow denotes the 43-bp *Nco*I-*Bss*HII fragment (with the arrow indicating the direction of the insert), and arrow above the boxes indicates the transcription initiation site. (A) 5′ end analysis. The 5′ end of RNA transcripts was determined by primer extension analysis by using a 20-nucleotide oligonucleotide complementary to the major late transcription unit of adenovirus and the polylinker. The sizes of the primer and the extended products are indicated. (B) Northern analysis. The same oligonucleotide as in primer extension analysis was used to detect RNA transcripts generated in injected oocytes. The short transcripts (ST) and read-through transcripts (RT) are indicated. Lanes: 1, TERr injection; 2, TERc injection. (C) The DNA sequence at the 5′ end of the human ADA gene that mediates transcription arrest. The sequence of the 43-bp *Nco*I-*Bss*HII fragment is shown, and the exon-intron boundary is marked. A bracket indicates the position corresponding to the 3′ end of short transcripts. The nucleotides underlined represent those positions in exon 1 where the human and murine ADA gene differ. In all four cases, the nucleotide present in the murine ADA gene is an A residue.

valid and informative. A cell-free transcription system using HeLa nuclear extracts has been developed in our laboratory to study transcriptional arrest in the murine ADA gene (23). In this cell-free system, prematurely terminated transcripts are similar to those detected in injected oocytes (23, 41), indicating that basic aspects of the exon 1 termination events are conserved in both transcription systems. Others have extensively studied exon 1 transcriptional arrest of the proto-oncogene c-*myc* both in injected oocytes and in a cell-free transcription system (7, 11, 32). Major conclusions drawn from the two different approaches are generally consistent. Therefore, the *Xenopus* oocyte transcription system appears to reproduce exon 1 transcriptional arrest events that occur in mammalian cells. Because the human ADA promoter does not function efficiently in the in vitro transcription system (22a), we have continued to use the oocyte transcription system for assaying the human ADA exon 1 termination process. Although factors required to regulate transcriptional arrest may not be present in *Xenopus* oocytes, the oocyte transcription system appears to serve as a suitable assay to study basic features of the exon 1 transcriptional arrest process.

The 43-bp DNA fragment of the ADA gene identified here does not share significant homology with other eukaryotic DNA sequences that have been documented to block transcription elongation. In several viral transcription units, the sites of premature transcription termination consist of sequences with the potential to form a GC-rich stem-loop structure in the RNA, followed by a uridine stretch (3, 20, 27, 46). Similarly, the transcription block sites in c-*myc* genes also encode potential stem-loop structures followed by a stretch of U residues (7). Previously, we showed that an ADA minigene construct is sufficient to encode the 105-nucleotide RNA, indicating that intron 1 sequences are not required for transcriptional arrest (8). Therefore, the DNA sequences important for the transcriptional arrest signal in the human ADA gene can be further narrowed down to a 34-bp DNA sequence at the end of exon 1 (Fig. 5C). The 34-bp DNA sequence does not have an obvious stem-loop structure or a long consecutive stretch of T residues at the termination region. A similar exon 1 transcription block has also been detected in the murine ADA gene (41). Comparison of the human and murine ADA transcriptional arrest regions reveals that, within the 3′ end of exon 1, they share 30 identical nucleotides and differ only at 4 positions (Fig. 5C). We have not found significant homology between the ADA transcriptional arrest signal and other premature termination signals reported. The transcripts generated from exon 1 of the ADA genes do not seem to form an attenuation-like structure. It is conceivable that the exon 1 transcriptional arrest signal of the ADA genes represents a novel type of premature termination of transcription.

Mechanistically, how is the exon 1 transcriptional arrest signal recognized? On the basis of the following evidence, it is possible that the transcriptional arrest signal of the ADA gene is recognized at DNA level. First, the arrest signal could be narrowed down to a 34-bp DNA fragment near the end of exon 1. Deletion of DNA sequences downstream of the 3′ ends of the short transcripts abolished transcriptional arrest in both injected oocytes and the cell-free transcription system (41; unpublished observations), indicating that DNA sequence downstream of the arrest site is critical. Second, the addition of extra sequence at the 5′ end of the ADA transcripts did not alter the transcriptional arrest (Fig. 3), suggesting that, under these circumstances, the length and composition of the 5′ leader sequence were not essential for the arrest. Third, the DNA fragments immediately downstream of both murine and human ADA gene transcriptional arrest sites demonstrated specific binding of protein factor(s) present in HeLa nuclear extract (22a). Although the linkage between the protein binding to the DNA sequences and the transcriptional arrest process remains to be determined, it is possible that the binding of protein factor(s) in this region plays a role in transcriptional arrest. In this regard, we noted

that the exon 1 transcriptional arrest signal is recognized by RNA polymerases II and III. This finding is consistent with the presence of a DNA binding protein that impedes the progression of RNA polymerase. Specific binding of protein factors to stop transcription has been found for RNA polymerase I (29, 35), mitochondrial RNA polymerase (10, 12), and the CCAAT region of the adenovirus major late promoter (14, 15).

How could the transcriptional arrest signal be overcome to achieve high-level expression of the ADA gene such as in Molt-4 cells? In other words, how could transcriptional arrest be regulated? First, events occurring at transcription initiation may govern termination. In several eukaryotic genes—for instance, in human U1 and U2 small nuclear RNA genes (21, 40) and the c-*myc* genes (7, 37, 54)—transcription termination is dependent on certain promoters. These findings suggest that the transcription complex assembled at the promoter region may be predetermined in such a way that it will either (i) respond to transcription termination signals downstream to cease transcription or (ii) ignore transcription termination signals to allow transcription readthrough. Alternatively, another mechanism to control transcription elongation is to modify RNA polymerase complexes to a terminating or antiterminating form by accessory factors. A well-characterized example is represented by the control of lambda early gene expression by the antitermination protein N (2, 22). Another example is provided by the effect of Tat on the IST element of the HIV-1 long terminal repeat. Tat may direct formation of polymerase II transcription complexes capable of efficient transcription elongation by functioning as a gene-specific transcription elongation factor (17, 42). These studies substantiate the notion that transcription complexes can either be assembled differentially at the promoter region or modified early after transcription initiation so that they will respond to downstream transcription termination signals accordingly. Therefore, we propose that a T-cell-specific enhancer identified in intron 1 of the ADA gene (1) may be involved in the antitermination mechanism to promote transcription through the exon 1 transcriptional arrest site. Although the role of enhancers at the molecular level remains unsolved, it is generally believed that enhancers can function over a long distance to influence the formation or the activity of the transcription complex. The T-cell-specific enhancer of the ADA gene may facilitate the formation of a transcription complex that is capable of reading through the transcriptional arrest signal. In fact, it has been shown recently that when enhancer elements are inserted into the 5′ flanking region of the human c-*myc* gene, premature termination in exon 1 is greatly repressed in transfected cells (5). Thus, we postulate that tissue-specific expression of the ADA gene is governed by factors that function as antiterminators to determine the fate of the transcription complex as it travels into the exon 1 arrest signal. Under most circumstances, transcription complexes initiated from the ADA promoter will be blocked within exon 1. In some cells (e.g., Molt-4), an antitermination mechanism will promote transcription complexes to read through the arrest site, resulting in the synthesis of full-length ADA transcripts. Likely candidates for such antitermination proteins are any that specifically associate with the ADA intron 1 enhancer. These proteins may promote the assembly of a transcription complex that is immune to the exon 1 transcriptional block signal. This antitermination model does not contradict the possibility that termination factors may bind at the arrest site to mediate transcriptional arrest, as discussed earlier. One can envision that an enhancer-binding protein might somehow disrupt the function of the putative termination factor. Since we have delineated the *cis*-acting element responsible for the transcriptional arrest, this model is under investigation.

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