# Transcriptional elongation of the rat apolipoprotein A-I gene: identification and mapping of two arrest sites and their signals

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**Abstract** **Previous studies have shown that the elongation phase of apoA-I gene transcription is regulated and contributes to hormone-induced changes in the expression of this gene in rat liver. We have now identified, by in vitro transcription studies with HeLa nuclear extracts, two transcriptional arrest sites within exon 3 and intron 3, respectively. Two truncated transcripts of 510 and ~1100 nucleotides in length, termed attenuator 1 RNA and attenuator 2 RNA, respectively, were observed when a rat apoA-I genomic fragment extending from −309 to +1842 relative to the transcription start site was transcribed in vitro in the presence of KCl or Sarkosyl. The attenuation events were promoter-independent as transcription of the apoA-I gene driven by the cytomegalovirus promoter resulted in transcriptional arrest at both sites. Transcription studies using deletion constructs as templates identified nucleotides +976 to +1158 as a region that contained the signal for transcriptional arrest at attenuator site 2. Computational analysis predicted a stem–loop structure in the nascent RNA immediately upstream of the arrest site. Deletion of attenuator 2 signal or deletion of sequences +147 to +216 located far upstream of the actual elongation block site 1 abrogated arrest at site 1. Thus, complex long-range interactions may be involved in the transcriptional arrest at site 1.** **These elongation blocks identified in vitro are consistent with earlier in vivo data based on nuclear run-on assays and represent, to our knowledge, the first example describing transcriptional attenuation as a mechanism controlling the expression of a member of the apolipoprotein gene family.**—Dallinger, G., H. Oberkofler, C. Seelos, and W. Patsch. **Transcriptional elongation of the rat apolipoprotein A-I gene: identification and mapping of two arrest sites and their signals.** ***J. Lipid Res.*** **1999.** 40: **1229–1239.**

**Supplementary key words** apolipoprotein A-I • transcriptional attenuation • elongation block • in vitro transcription

The primary control point in the regulation of differential gene expression is the frequency of transcription initiation which depends on the interaction of transcription factors with promoter or enhancer elements (1, 2). In addition to transcription initiation, regulation of gene expression at the level of transcript elongation is well established in prokaryotes (for review, see 3, 4) and an increasing body of evidence indicates that attenuation events also contribute to the regulation of gene expression in eukaryotes. Early studies described viral transcription units including adenovirus type 2 (5, 6), simian virus 40 (7), polyomavirus (8), or human immunodeficiency virus (9, 10) as paradigms of transcription attenuation events in eukaryotes. More recently, control at the level of transcript elongation has also been observed in several eukaryotic genes themselves. Blocks to transcription elongation regulate the abundance of proto-oncogenes such as c-myc (11–16), c-myb (17, 18), or c-fos (19–21) and other genes such as adenosine deaminase (22–26), ornithine decarboxylase (27), histone 3.3 (28), or drosophila heat shock genes (29). Eukaryotic DNA sequences representing transcription blocks have been identified (12, 13, 15, 21, 22, 25, 27, 28), but these sequences are not the sole determinants of regulating read-through as termination can be modulated by initiation from different promoters. Hence, modification of the transcription complex by accessory factors may alter the processivity of RNA polymerase, and thus determine termination or read-through. In addition, RNA secondary structure may play a critical role in attenuation events (6, 30, 31).

Apolipoprotein (apo) A-I, the main apolipoprotein of HDL, is involved in the initial phase of reverse cholesterol transport (32, 33) which may, at least in part, account for its protective role against atherosclerotic disease (34). Several metabolic signals have been shown to influence apoA-I gene expression (35–38). In rats, chronic administration of thyroid hormone ($T_3$) increased plasma levels of apoA-I and the level of mature nuclear and cytoplasmic

Abbreviations: apo, apolipoprotein; CMV, cytomegalovirus; EDTA, ethylenediaminetetraacetic acid; HDL, high density lipoprotein; RT-PCR, reverse transcription-polymerase chain reaction; SOE, splice-overlap extension; $T_3$, triiodothyronine.

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apoA-I mRNA in liver, even though apoA-I gene transcription as well as the abundance of primary transcripts was reduced (36). In vivo data on transcript processing indicated that chronic $T_3$ treatment of rats enhanced apoA-I mRNA maturation severalfold by protecting the mRNA precursor devoid of intron 2, but containing introns 1 and 3 from degradation and/or facilitating the splicing of intron 1 from this precursor (39). As a result, apoA-I mRNA expression in liver was increased despite reduced mRNA synthesis. To gain further insight into the inverse association between rates of apoA-I mRNA maturation and synthesis of primary transcripts, transcription across the apoA-I gene was measured by nuclear run-on with single-stranded overlapping RNA probes. These studies showed no difference in the rate of transcript initiation between control and hyperthyroid rats, but revealed transcriptional elongation block(s) that were twice as effective in $T_3$-treated rats in comparison to controls (40). To gain further insight into the potential role of transcriptional arrest in apoA-I gene expression, we mapped the location of the elongation block(s) more precisely using cell-free transcription of apoA-I templates in HeLa nuclear extracts.

## MATERIALS AND METHODS

### Construction of apoA-I templates

A 2152 bp apoA-I DNA fragment as well as a 1135 bp ΔapoA-I fragment were amplified by polymerase chain reaction (PCR) starting from a rat genomic clone containing the entire AI/CIII/A-IV gene cluster (41) using 5′ ATGAGACTAACTAAAAGAAAC 3′ (−309 to −288) as upper primer and 5′ TCCCTTGATGTCC CCTTAGC 3′ (+1842 to +1822) or 5′ CTGGGGGTTACCAAGG AAAAG 3′ (+826 to +805) as lower primers, respectively. The numbers in parentheses designate the 5′ and 3′ ends in the genomic DNA relative to the transcription start site. All primers were synthesized using a Beckman Oligo 1000 DNA Synthesizer (Beckman Instruments Inc., Fullerton, CA). The PCR reactions contained 0.2 μm of each upstream and downstream primer, 200 μm of dNTP, 10 mm Tris-HCl, pH 8.3, 50 mm KCl, 2.5 mm $MgCl_2$, and 2.5 units of Pfu-DNA polymerase (Stratagene, La Jolla, CA) in a 100 μl reaction volume that was overlaid with mineral oil. Samples were processed through initial denaturation for 3 min at 95°C followed by 30 cycles of amplification each consisting of 1 min annealing at 56°C (cycles 1–3), at 53°C (cycles 4–6), and at 50°C (cycles 7–30), 1 min extension at 72°C, 1 min denaturation at 95°C, and a final extension at 72°C for 5 min. The resulting PCR fragments were cloned into pGEM-3Zf (Promega Corp., Madison, WI) and the sequence of inserts was verified by dye terminator cycle sequencing using an ABI PRISM™ 310 Genetic Analyzer (Perkin Elmer-Applied Biosystems, Foster City, CA).

Deletion mutants were constructed by splice-overlap extension (SOE)-PCR (42) starting from the 2152 bp apoA-I construct described above. The SOE-upper and -lower primers for the different deletion constructs are given in **Table 1**. Primer pairs for the first round of PCR amplifications were 5′ TAATACGACTCACT ATAGGG 3′ (T7 primer) and the respective SOE-lower as well as 5′ TATTTAGGTGACACTATAG 3′ (SP6 primer) and the respective SOE-upper primers. PCR conditions were as described for the apoA-I construct. Overlapping PCR products were gel purified, mixed in equimolar amounts, and allowed to anneal. Extension of single strands was performed with 2.5 units Pfu-DNA polymerase at 72°C for 4 min. Extended strands were amplified using T7 and SP6 primers and the PCR conditions described above. All SOE-PCR fragments were cloned into pGEM-3Zf using Kpn I (3′) and BamH I (5′) and sequences of inserts were verified. For the cytomegalovirus (CMV)–apoA-I construct, the CMV promoter from the plasmid pRc/CMV2 (Invitrogen, Carlsbad, CA) was linked to the apoA-I coding sequence by SOE-PCR. Primers for the first round of PCR amplification were 5′ *CGGGATCC*ATA TACGCGTTGACATTG 3′ as upper primer with a BamHI site in italics and 5′ CTCTCCAACAGTTAGCCAGAGAGCTCTGCT 3′ as SOE-lower primer and 5′ TCTGGCTAACTGTTGGAGAGCTCC GGGGGAG 3′ as SOE-upper primer and the T7 primer as lower primer, respectively. Purified overlapping PCR products were extended and PCR-amplified with Pfu-DNA polymerase using the CMV-upper and T7 primer, respectively, and the reaction conditions described above except for annealing temperatures of 58°C (cycles 1–5), 55°C (cycles 6–10), and 52°C (cycles 11–35). The resulting PCR fragment was cloned into pGEM-3Zf and sequenced.

### In vitro transcription assays

The full-length apoA-I plasmid as well as all deletion plasmids and the CMV–apoA-I plasmid were digested with BamHI and Kpn I. The 3′ protruding ends generated by the Kpn I restriction were filled in using T4 DNA-polymerase (New England Biolabs, Beverly, MA) in a reaction mix containing 10 mm Tris-HCl, pH 7.9, 10 mm $MgCl_2$, 50 mm NaCl, 1 mm DTT, 1 mm dNTPs, and 12 units T4 DNA polymerase. The reaction was in-

TABLE 1. SOE primers for the construction of apoA-I deletions

| Construct | SOE-Upper Primer | SOE-Lower Primer |
|---|---|---|
| Δ1 | 5′***AGGCACCCAC***TAGCAGCACTTACCTCCCCC 3′ (+18, +38) | 5′ ***AGTGCTGCTA***GTGGGTGCCTCTTGGTCTCC 3′ (+286, +306) |
| Δ1.1 | 5′ ***AGAATGGCTG***TAGCAGCACTTACCTCCCCC 3′ (+18, +38) | 5′ ***AGTGCTGCTA***CAGCCATTCTTCTTCTTCTT 3′ (+199, +219) |
| Δ1.2 | 5′ ***AGGCACCCAC***CCACACACATTCAACCCAGT 3′ (+147, +167) | 5′ ***ATGTGTGTGG***GTGGGTGCCTCTTGGTCTCC 3′ (+286, +306) |
| Δ2 | 5′ ***CTGTCCCATT***GATGTGGGCGACCTGGAGAA 3′ (+216, +236) | 5′ ***CGCCCACATC***AATGGGACAGGGTGAAGGAT 3′ (+483, +503) |
| ΔAtt.2 | 5′ ***CCCAGTTGTC***CTCCCTTCCCATCCCTCTGT 3′ (+956, +976) | 5′ ***GGGAAGGGAG***GACAACTGGGACACTCTGGG 3′ (+1158, +1177) |
| ΔAtt.2.1 | 5′ ***AAAAGGGATT***TAGGGTAGGTAGCGCACCTT 3′ (+27, +47) | 5′ ***ACCTACCCTA***AATCCCTTTTCCTTGGTAAC 3′ (+798,+818) |
| ΔAtt.2.2 | 5′ ***TTCTGTTTCA***GAGCAGCTGAGAGATGACCC 3′ (+1110, +1130) | 5′ ***TCAGCTGCTC***TGAAACAGAAGATGCAGCCC 3′ (+1295, +1315) |

The numbers in parentheses refer to nucleotides in the genomic DNA relative to the transcriptional start site. Nucleotides in bold or in italics refer to the overlapping regions of the SOE primers.

cubated at 11°C for 20 min followed by an inactivation step at 75°C for 10 min. DNA fragments were gel-purified and eluted using the Nucleotrap gel elution kit (Macherey-Nagel, Düren, Germany).

Cell-free in vitro transcription of apoA-I RNA was performed using nuclear extracts from HeLa cells (GIBCO-BRL Life Technologies, Grand Island, NY). Template DNA (250–300 ng) was pre-incubated for 10 min with 5 μl (7.03 mg/ml) of nuclear extract, 6 μl transcription buffer containing 20 mm HEPES, pH 7.9, 100 mm KCl, 0.2 mm EDTA, 0.5 mm DTT, and 20% (v/v) glycerol, 3 mm $MgCl_2$ and 60 units RNAsin (Promega Corp.). A pre-incubation without NTPs to allow transcription complex formation was followed by a 1 min pulse with 400 μm each of rATP, rCTP, and rGTP, and 3 μCi [$\alpha^{32}$P]UTP (3000 Ci/mmol, Amersham Life Science, Buckinghamshire, UK). After addition of 400 μm rUTP and up to 0.8 m KCl or up to 1.25% Sarcosyl, the in vitro transcription reaction was allowed to proceed for 30 min at 30°C. The reaction was terminated by addition of 200 μl of 0.3 m Tris-HCl, pH 7.4, 0.3 m sodium acetate, 0.5% SDS, 2 mm EDTA, 3 μg/ml tRNA, and 10 μl (10 mg/ml) Proteinase K (Boehringer Mannheim GmbH, Mannheim, Germany). After digestion for 10 min at 37°C, the in vitro transcribed RNA was purified using Chromaspin 100-DEPC-columns (Clontech, Palo Alto, CA), ethanol precipitated, and separated in 4% denaturing polyacrylamide gels.

## Primer extension analysis

The oligonucleotide primer 5′ GGGAGGCATGAGGGAACTAA 3′ (+56 to +76) was gel-purified in 18% polyacrylamide gels and end-labeled using 10 units of T4 polynucleotide kinase (New England Biolabs) in a reaction mix containing 70 mm Tris-HCl, pH 7.6, 10 mm $MgCl_2$, 5 mm DTT, 2.5 μl [$\gamma^{32}$P]ATP (3000 Ci/mmol, Amersham Life Science) in a 10 μl total volume. Reactions were incubated at 37°C for 60 min and heat-inactivated at 95°C for 10 min. ApoA-I RNA was in vitro transcribed and template DNA was removed by digestion with 1 unit of RQ1 RNase-free DNase (Promega Corp.) at 37°C for 30 min followed by ethanol precipitation of the RNA products. Annealing of 10 μm $^{32}$P-labeled primer to in vitro transcribed RNA was performed in 9 μl hybridization solution containing 50 mm Tris-HCl, pH 8.3, 75 mm KCl, 3 mm $MgCl_2$, and 0.5 mm DTT. The reaction was overlaid with mineral oil, denatured at 95°C for 5 min, and allowed to cool down to 52°C. After incubation for 60 min, 66 units of M-MLV-reverse transcriptase (GIBCO-BRL Life Technologies), 1.5 μl of 0.1 M DTT, 1.5 μl actinomycin D (50 μg/ml), and 40 units RNasin were added and the mixtures were incubated at 42°C for 1 h. Primer extension products were phenol/chloroform extracted, ethanol precipitated, and separated by electrophoresis in 10% polyacrylamide–8 m urea gels.

## DNase I footprint analysis

A 466 bp genomic apoA-I fragment was amplified using the full-length apoA-I plasmid as template and 5′ GACTGTTGGAG AGCTCCG 3′ (−3 to +15) and 5′ CTCATCTTGCTGCCAGAA CT 3′ (+469 to +450) as upper and as lower primer, respectively. The upper or the lower primer was end-labeled as described to obtain a DNA duplex labeled on one strand. Nuclear extracts from rat hepatocyte as well as HeLa cells were prepared according to the method of Dignam, Lebovitz, and Roeder (43). Fifty μg protein of each extract was incubated with 3 μg poly (dI-dC), 10% glycerol, 15 mm HEPES, 50 mm KCl, 1 mm EDTA, 5 mm DTT, and 5 mm $MgCl_2$ in a total volume of 96 μl on ice for 15 min. After addition of 5 fmol (~25.000 cpm) of end-labeled DNA, the incubation was continued for 45 min, digested with 2 μl of DNase I solution (100 μg/ml, Boehringer Mannheim) for 5 min on ice, and stopped by the addition of 200 μl of a solution containing 100 mm Tris, pH 7.4, 100 mm NaCl, 1% SDS, and 10 mm EDTA. The mixture was digested with 100 μg of proteinase K (Boehringer Mannheim) for 30 min at 65°C, extracted with phenol/chloroform, and ethanol precipitated. Reaction products were applied to a 6% acrylamide gel containing 8 m urea along with a G+A sequencing reaction of the template (44).

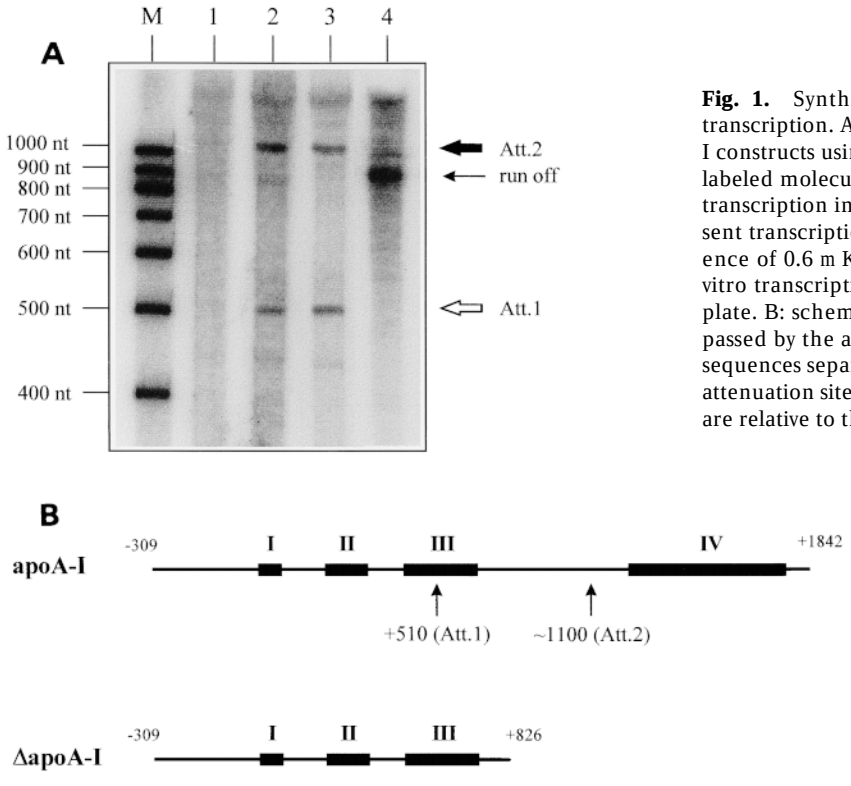



**Fig. 1.** Synthesis of two truncated apoA-I transcripts by in vitro transcription. A: in vitro transcription of the rat apoA-I and ΔapoA-I constructs using HeLa nuclear extracts. M refers to a radioactively labeled molecular weight marker. Lane 1 shows results of a control transcription in the absence of template DNA. Lanes 2 and 3 represent transcription reactions using the apoA-I construct in the presence of 0.6 m KCl or 0.7% Sarkosyl, respectively. Lane 4 shows in vitro transcription products using the ΔapoA-I construct as a template. B: schematic representation of the genomic region encompassed by the apoA-I and ΔapoA-I constructs. Boxes indicate exon sequences separated by introns shown by lines. The positions of the attenuation sites (Att.1 and Att.2) are indicated by arrows. Numbers are relative to the transcription start site.

### RNA structure analysis

RNA-folding was analyzed using an RNA secondary structure prediction algorithm described by Zuker, Jaeger, and Turner (45).

## RESULTS AND DISCUSSION

After optimization of the in vitro transcription reaction with respect to nucleotide, template and $MgCl_2$ concentration, initial experiments were directed towards detection of elongation blocks in apoA-I transcripts. It is well established that high concentrations of KCl and/or Sarkosyl, an anionic detergent, cause transcription attenuation at specific elongation block sites which correspond to attenuation sites identified in vivo (5, 46–48). Both KCl and Sarkosyl are thought to dissociate elongation factors from the transcription complex thereby reducing the processivity of the RNA polymerase II (27, 46, 49). We therefore performed in vitro transcription experiments using a full-length apoA-I template DNA spanning nucleotides −309 to 1842 in the presence of 0.3 to 1.2 m KCl and observed two incomplete transcripts of 510 and ~1100 nucleotides. Inclusion of up to 1.0% Sarkosyl in the transcription reaction produced similar results. Transcripts of 510 and ~1100 nucleotides were reproducibly obtained in several independent assays at 0.6 m KCl or 0.7% Sarkosyl (**Fig. 1A**) and were therefore designated attenuator 1 RNA and attenuator 2 RNA, respectively. Addition of KCl as well as Sarkosyl in the same reaction produced no difference in the signal intensity ratio of the two transcripts (not shown). These initial experiments were consistent with previous nuclear run-on studies suggesting transcription elongation block(s) within a region extending from +449 to +1086 (40).

Full-length apoA-I transcripts were not clearly visible under these conditions. Similarly, full-length transcripts were also not clearly detectable when KCl or Sarcosyl was omitted from the transcription reaction (not shown). Several factors may have contributed to this result. First, background signals resulting from transcription of residual DNA templates in HeLa extracts were observed in the respective gel region and may have concealed the identification of full-length transcripts. Second, our in vitro system may have been incompetent for faithful synthesis of an 1.8 kb RNA. Third, the elongation blocks located in exon 3 and intron 3, respectively, may have reduced transcription of downstream sequences to such an extent that the level of full-length transcripts was below the detection limit. To demonstrate that our transcription system was competent for full-length transcription from the apoA-I promoter, we used an apoA-I template that was truncated at nucleotide +826 (ΔapoA-I) and observed newly synthesized RNA of a size that was expected for run-off transcripts (Fig. 1A). Interestingly, no attenuator 1 RNA was detectable in transcriptions from the truncated template (see below).

To verify that transcription initiation occurred in vitro at the authentic transcription start site of the apoA-I gene, primer extension analysis was performed. A $^{32}$P-labeled primer complementary to nucleotides +56 to +76 of the apoA-I primary transcript was hybridized to in vitro transcribed RNA followed by an extension reaction at 42°C. An extension product of 76 nucleotides was observed in denaturing gels indicating correct transcription start site usage (**Fig. 2**). A shorter extension product of ~48 nt probably reflecting pausing of reverse transcriptase was also detectable, but extension products consisting of more than 76 nucleotides were not observed in any of several primer extension experiments. Primer extension assays were also performed using apoA-I RNA templates transcribed in the presence of 0.6 m KCl or 0.7% Sarkosyl. These experiments showed no influence of KCl or Sarkosyl, at the concentrations indicated, on initiation site usage (Fig. 2).

To investigate possible promoter influences on elongation blocks in the rat apoA-I gene, the authentic apoA-I promoter was replaced by the CMV promoter. The CMV promoter sequence comprising nucleotides −609 to +1 (50) was linked to the apoA-I gene sequence +32 to +1842 by SOE-PCR. In vitro transcription reactions per-

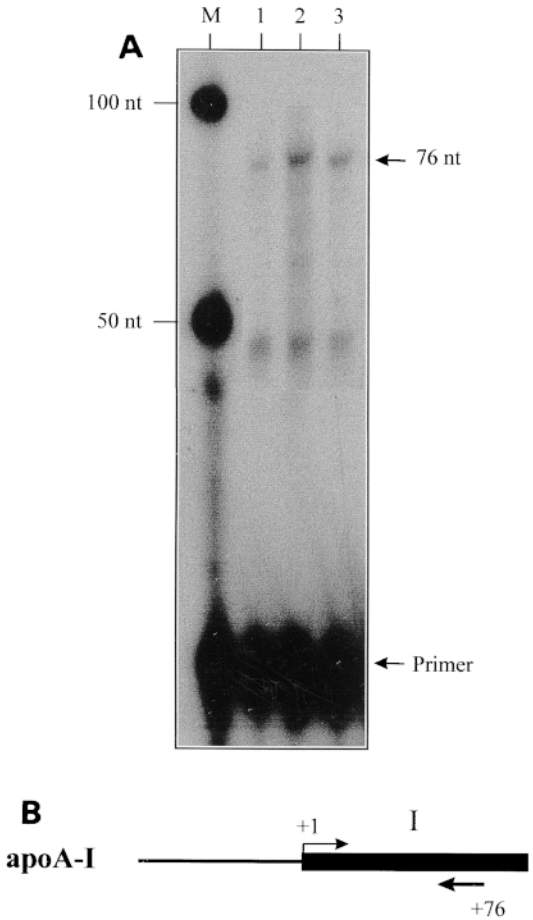



**Fig. 2.** Primer extension analysis indicates proper initiation site usage of in vitro transcribed apoA-I transcripts. A: M refers to a molecular weight marker. ApoA-I RNA transcribed in vitro in the absence of KCl or Sarkosyl was used as a template for the extension reaction shown in lane 1. In lanes 2 and 3, 0.6 m KCl or 0.7% Sarkosyl was included in the in vitro transcription reaction prior to primer extension. B: position of the primer used for primer extension studies relative to the transcription start site.

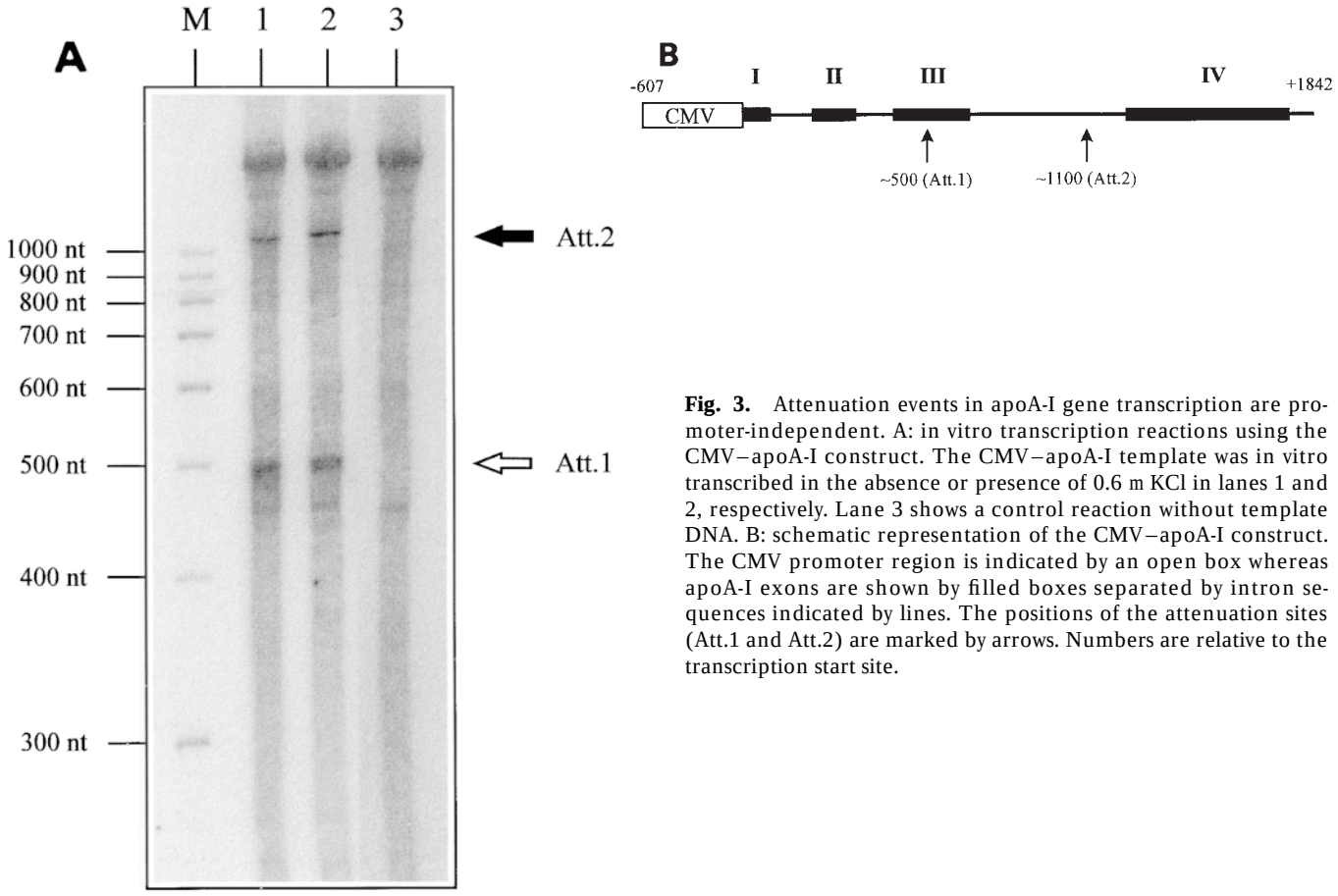
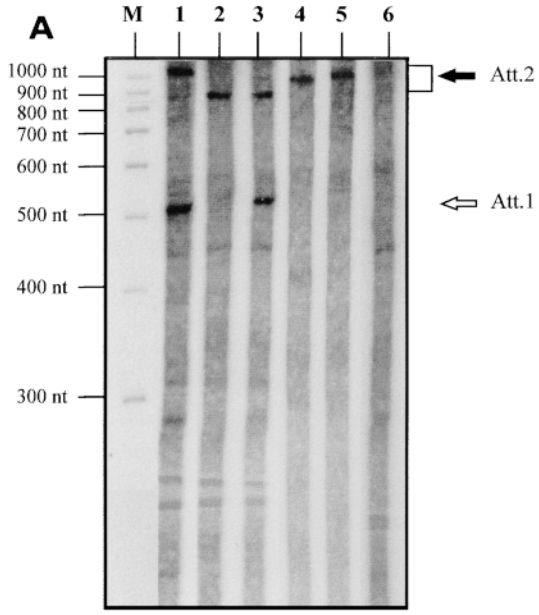



**Fig. 3.** Attenuation events in apoA-I gene transcription are promoter-independent. A: in vitro transcription reactions using the CMV–apoA-I construct. The CMV–apoA-I template was in vitro transcribed in the absence or presence of 0.6 m KCl in lanes 1 and 2, respectively. Lane 3 shows a control reaction without template DNA. B: schematic representation of the CMV–apoA-I construct. The CMV promoter region is indicated by an open box whereas apoA-I exons are shown by filled boxes separated by intron sequences indicated by lines. The positions of the attenuation sites (Att.1 and Att.2) are marked by arrows. Numbers are relative to the transcription start site.

formed with the CMV–apoA-I chimeric construct as a template showed two truncated transcripts corresponding to attenuator 1 and attenuator 2 RNA described. Neither Sarcosyl nor KCl was necessary to detect the elongation blocks in RNA transcribed from the chimeric template (**Fig. 3**). This result may be attributable to an imbalance of accessory factors present in the HeLa nuclear extracts and the strong CMV promoter activity resulting in an increased transcriptional efficiency. Nevertheless, these experiments clearly indicated that the two attenuation events across the apoA-I gene are, at least qualitatively, promoter-independent.

To determine the 5′ and 3′ boundaries of DNA region(s) responsible for the generation of the attenuator 1 RNA, several apoA-I clones harboring deletions between nucleotides +38 to +483 relative to the transcription start site were created by SOE-PCR (Table 1, **Fig. 4B**). Deletion of nucleotides +38 to +286 (construct Δ1) resulted in loss of attenuator 1 RNA (Fig. 4A), whereas attenuator 2 transcripts of the predicted size were observed. Interestingly, transcription from a deletion construct that spanned nucleotides +236 to +483 (construct Δ2) and was located closer to the arrest site 1 gave rise to two truncated transcripts. In addition to the expected attenuator 2 RNA, transcripts of ~500 nucleotides and therefore similar in size to attenuator 1 RNA were observed. The mechanism(s) underlying the synthesis of the ~500 transcripts from the Δ2 template are not clear, but the relationship between template deletions and the synthesis of attenuator 1-sized RNA suggested that an attenuation signal was contained, at least in part, within the sequence deleted in the Δ1 construct. Moreover, it may be postulated that this attenuation signal modified the transcription machinery to continue RNA synthesis for a distance of 250 to 300 nucleotides, while the sequences at the actual arrest site were probably of minor importance for the formation of attenuator 1 RNA.

To more precisely map the region essential for the elongation block generating attenuator 1 RNA, two templates with overlapping deletions contained within the Δ1 template (Δ1.1 and Δ1.2) were constructed (Fig. 4B). In vitro transcription of both these DNA templates resulted in a loss of attenuator 1 transcripts, whereas attenuator 2 RNA was synthesized in both reactions (Fig. 4A). The possibility that DNA–protein interactions could have contributed to attenuation at site 1 was studied by DNase I footprinting. Two protein-binding sites located at nucleotides +126 to +144 and +155 to +185, respectively, were identified using rat liver nuclear extracts (**Fig. 5A**). The presence of proteins bound to the respective regions was verified in competition experiments using a 500-fold molar excess of template DNA (Fig. 5B). Identical footprints were observed when HeLa nuclear extract was used instead of the rat nuclear extract (Fig. 5C). Both sequences protected in the DNase I footprint assays were deleted in the Δ1 and the Δ1.1 constructs, while only one footprint was deleted



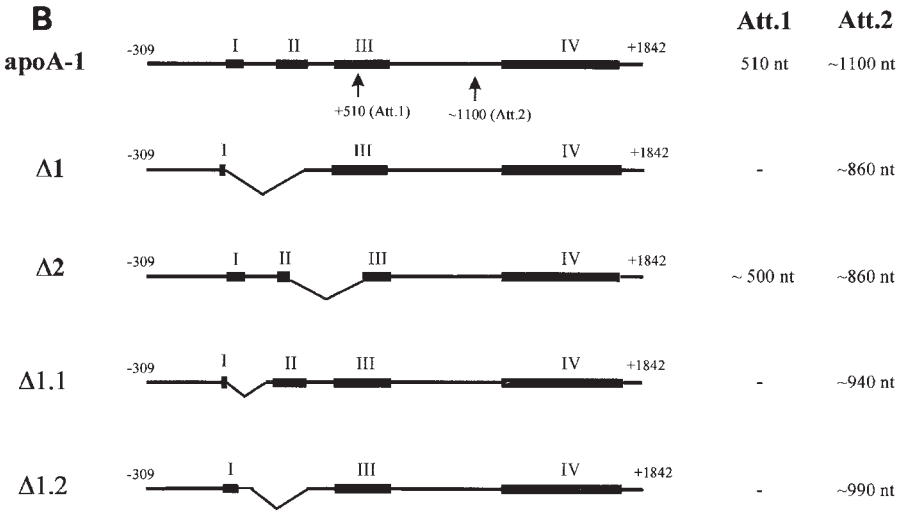




**Fig. 4.** Mapping of an attenuator 1 signal by in vitro transcription using deletion constructs as templates. A: the apoA-I template and several deletion constructs shown in B were in vitro transcribed in the presence of 0.6 M KCl using HeLa nuclear extracts. M refers to a molecular weight marker. Lanes 1–5 show in vitro transcription reactions of the original apoA-I construct and the deletion constructs Δ1, Δ2, Δ1.1, and Δ1.2, respectively. Lane 6 shows a control reaction without template DNA. The position of the elongation blocks is indicated by an open arrow (attenuator 1) or by a filled arrow (attenuator 2). B: structure of the apoA-I constructs used in these experiments. Exons are indicated by boxes separated by introns shown as lines whereas deletions are indicated by gaps. The length of the attenuated transcripts obtained in the in vitro transcription reactions is indicated on the right.

in construct Δ1.2 (**Fig. 6**). Hence, at least one of the respective nuclear proteins may be necessary for the attenuation event that resulted in the formation of attenuator 1 transcripts, even though the identified DNA contacts of these proteins were far upstream of the actual arrest site. Several studies have already shown that eukaryotic attenuation signals might be located upstream, downstream, or at the actual site of RNA polymerase II transcriptional blocks (27, 51), and DNA-binding proteins have been suggested to represent or contribute to arrest signals for some elongation blocks (52–54).

In an attempt to delineate the boundaries of the attenuator 2 signal, a region spanning nucleotides +976 to +1158 was deleted in construct ΔAtt.2 (**Fig. 7C**). An additional deletion was introduced in exon 4 (+1438 to +1843) downstream of the attenuator 2 site to monitor the adequacy of in vitro RNA synthesis by the presence of full-length transcripts of 1257 nt. Indeed, transcription from the ΔAtt.2 construct produced run-off transcripts of the predicted size, whereas attenuator 2 RNA was not detectable (Fig. 7A). The shorter band of ~590 nt in Fig. 7A must have represented nonspecific transcripts, as a band of similar size was also present in control reactions without template DNA. To test whether downstream sequences are involved in the attenuation event at site 2, a construct (ΔAtt.2.2) harboring deletions from nucleotides +1130 to +1295 and from +47 to +798, were used for in vitro transcription. Both the ΔAtt.2.2 and a control construct carrying only the proximal deletion (+47 to +798) (Att.2.1) gave rise to full-length transcripts of the expected size. Furthermore, attenuator 2 RNA was clearly detectable upon in vitro transcription of the ΔAtt2.1 and ΔAtt.2.2 constructs Fig. 7B). Thus, the attenuation signal responsible for the generation of attenuator 2 transcripts was located in close proximity to the actual arrest site of the polymerase complex and sequences downstream of the attenuator 2 site had probably no influence on transcriptional arrest.

The absence of attenuator 1 RNA in these experiments was an unexpected finding (Fig. 7A), but was consistent with earlier data showing that attenuator 1 RNA was not

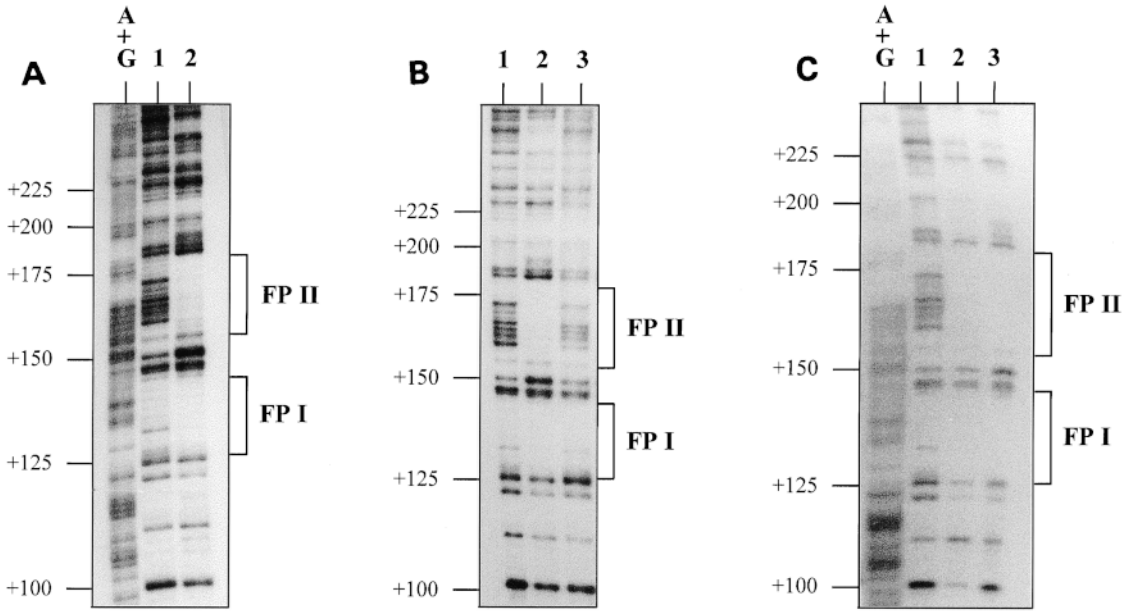



**Fig. 5.** A: DNase I footprint of two protein bindings sites, termed FP I and FP II, in the proximal rat apoA-I gene. A PCR-product that extended from −3 to +469 relative to the main transcription start was end-labeled on the coding strand of the duplex and used as a probe; lanes 1 and 2, shown next to an A/G sequencing ladder (A + G), represent separations of DNase I digests in the absence or presence of rat hepatocyte nuclear extract. B: DNase I digest in the absence of rat nuclear extract (lane 1) is compared with DNase I digests in the presence of rat nuclear extracts without competitor or with 500-fold molar excess of template (lanes 2 and 3). C: comparison of rat hepatocyte nuclear extract (lane 2) and HeLa nuclear extract (lane 3) binding; equal amounts of extract protein were incubated with template prior to DNase I digestion; lane 1 shows separation of a DNase I digest without nuclear extract next to an A/G sequencing ladder.

detectable after transcription from apoA-I templates truncated at +826 (Fig. 1). These experiments suggest an interaction of attenuator 2 or adjacent sequences with the attenuator 1 signal in that the former sequences are necessary for attenuation at site 1, but the latter sequences are not required for attenuation at site 2. This conclusion is also supported by the ΔAtt.2.1 and ΔAtt.2.2 construct experiments described above.

Sequences deleted in the ΔAtt.2 construct were analyzed for secondary structures by the method of Zuker et al. (45). These computations suggested that nucleotides +1040 to +1103, located immediately upstream of the arrest site 2, can form a strong RNA stem–loop structure (**Fig. 8**). Stem–loop structures followed by uracil-rich regions that form as the nascent RNA emerges from the transcription complex have been shown to cause or con-

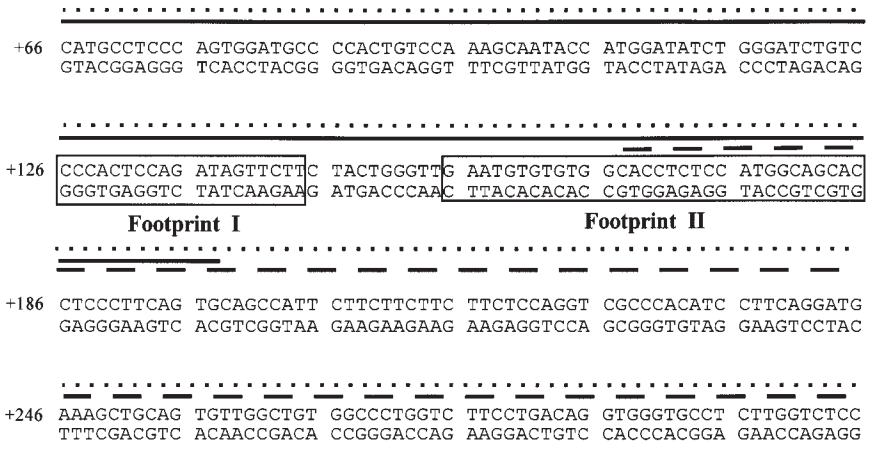



**Fig. 6.** Spatial relationship of an attenuator 1 signal to protein-binding sites in intron 1 of the apoA-I gene. DNA sequences protected during DNase I footprint analysis after incubation with HeLa nuclear extract are indicated by boxes. The numbers refer to the nucleotide positions relative to the transcription start site. The DNA region deleted in construct Δ1 is shown by dotted lines, whereas the regions deleted in Δ1.1 and Δ1.2 constructs are indicated by full lines and hatched lines, respectively.

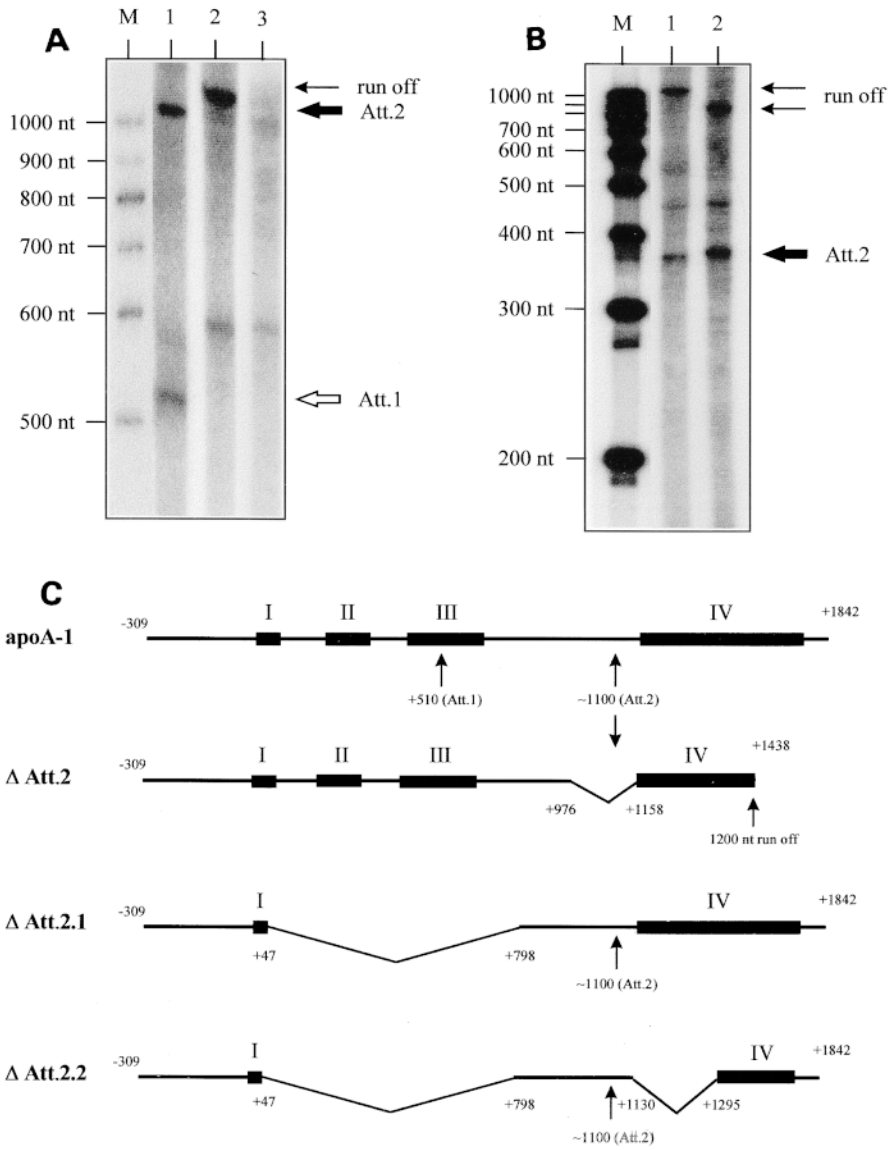



**Fig. 7.** Mapping of the attenuator 2 signal by in vitro transcription using deletion constructs as templates. A: cell-free in vitro transcription in the presence of 0.6 m KCl was performed using the original apoA-I construct and deletion construct ΔAtt.2 in lanes 1 and 2, respectively. Lane 3 shows a control reaction without template DNA. B: in vitro transcription was performed in the presence of 0.6 m KCl using the deletion constructs ΔAtt.2.1 and ΔAtt.2.2. In A and B, positions of full-length and attenuated transcripts are indicated by arrows. C: schematic representation of the apoA-I construct and the deletion constructs ΔAtt.2, ΔAtt.2.1, and ΔAtt.2.2. Exons are indicated by boxes separated by introns shown as lines whereas deletions are indicated by gaps. Numbers are relative to the transcription start site.

tribute to attenuation of transcript elongation in several genes (13, 55, 56). In the apoA-I gene, a uracil-rich region is not present downstream of the stem–loop structure, but deletion experiments in other genes have shown that stem–loop structures alone can induce attenuation events and uracil-rich regions may be dispensable in such a context (12). Therefore, the stem–loop of nascent apoA-I RNA may be sufficient to arrest the polymerase complex at site 2.

In this study we have localized two attenuation signals in the rat apoA-I gene using a HeLa-based cell-free transcription system. Experiments using a CMV-promoter/apoA I hybrid construct clearly demonstrated the promoter-independence of both arrest signals. The localization of the two attenuation signals is consistent with previous in vivo studies showing transcription elongation block(s) within this region (40). The correlation of the in vitro studies with earlier in vivo experiments together with the fact that HeLa cell nuclear extracts have been used extensively for the characterization of attenuation signals within other eukaryotic genes (14, 26, 27) argues for the validity of the transcriptional arrest sites described in the apoA-I gene. Nevertheless, possible modulatory effects of hepatocyte-specific nuclear proteins on apoA-I transcript elongation could not be ascertained because of the heterologous system used.

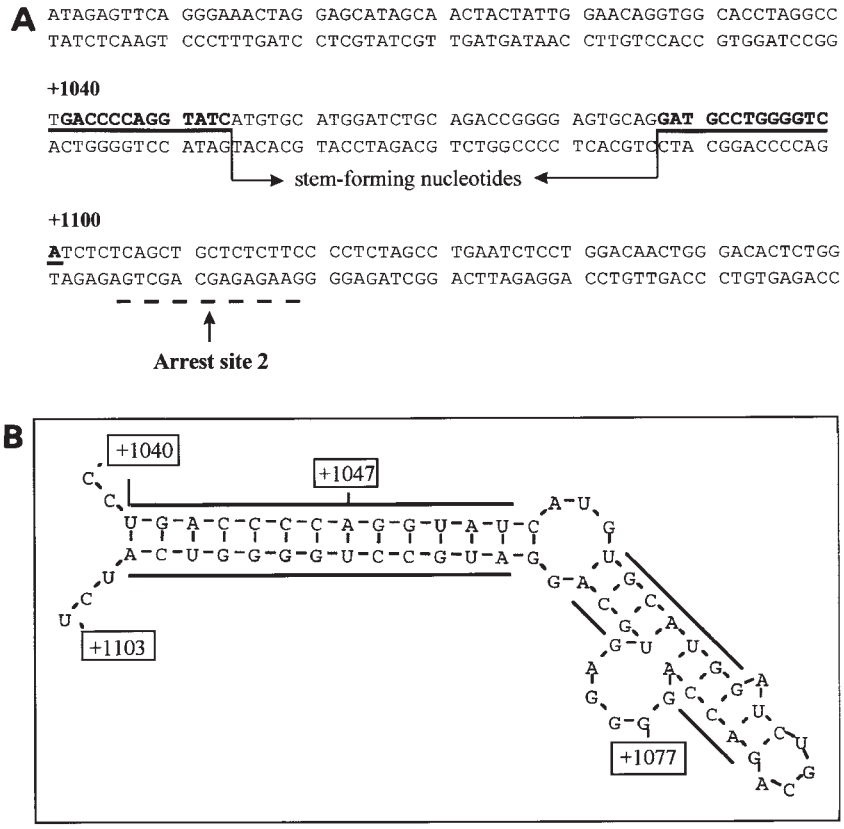



**Fig. 8.** Spatial relation of a putative stem–loop structure in the nascent RNA with the transcriptional arrest site 2. A: DNA region deleted in the Δ2 construct with the stem forming nucleotides (bold and underlined). Numbers are relative to the transcription start site. B: RNA secondary structure predicted by computational analyses using the algorithm of Zucker et al. (45).

The attenuation 2 site located in intron 3 fulfilled the criteria of classical attenuation sites. Deletion of sequences upstream of the attenuator 2 signal resulted in the synthesis of truncated transcripts of the expected size (Fig. 4). Mapping studies showed that the attenuation 2 signal was located in close proximity to the actual arrest site and computational analysis supports a functional role of a stem–loop structure that is located in the nascent RNA immediately upstream of the elongation block site. The mechanism(s) responsible for transcriptional arrest at the attenuation 1 site in exon 3 of the apoA-I gene appear to be more complex. Mapping studies showed that one region critical for the generation of attenuator 1 transcripts is located far upstream of the actual arrest site, while a second signal must be in close proximity to the attenuator 2 signal, implying a long-distance interaction. Long-distance interactions of attenuation signals have been described for the rat polymeric immunoglobulin receptor gene (51) and the murine ODC gene (27). Two short sequence stretches protected during DNase I footprint analysis are contained within one attenuator 1 signal and suggest a possible interaction of DNA binding proteins with the transcription complex.

To our knowledge, these and our previous studies (40) represent the first report describing transcriptional attenuation as a mechanism regulating the expression of a member of the apolipoprotein gene family. As apolipoprotein genes share extensive homologies, it is conceivable that the expression of other apolipoproteins may also be regulated by elongation blocks. ApoA-I is an antiatherogenic protein. Efforts to increase apoA-I gene expression represent, therefore, a rational approach for preventing atherosclerosis. Several studies focused on enhancement of apoA-I gene transcription (57–61). Our previous experiments demonstrated an inverse relationship between apoA-I mRNA synthesis and maturation rate with the latter being quantitatively far more important than the former, at least in rat liver (39). The factors regulating apoA-I transcript elongation may therefore be affected by or contribute to the mRNA maturation rate, and knowledge of the molecular mechanisms involved may suggest experimental strategies that permit dissociation of transcript elongation and mRNA maturation, thereby augmenting apoA-I gene expression. Even though our current studies were obtained in an in vitro transcription system consisting of heterologous components, our results represent a first step to delineate these processes. Clearly, in vivo studies will be required to substantiate our observations, to gain further insight into the physiological significance of the two attenuation signals, and to determine whether mRNA maturation affects transcriptional attenuation as was suggested by our earlier studies.

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## REFERENCES

1. Novina, C. D., and A. L. Roy. 1996. Core promoters and transcriptional control. *Trends Genet.* **12:** 351–355.
2. Tjian, R., and T. Maniatis. 1994. Transcriptional activation: a complex puzzle with few easy pieces. *Cell.* **77:** 5–8.
3. Platt, T. 1986. Transcription termination and the regulation of gene expression. *Annu. Rev. Biochem.* **55:** 339–372.
4. Friedman, D. I., M. J. Imperiale, and S. L. Adhya. 1987. RNA 3′ end formation in the control of gene expression. *Annu. Rev. Genet.* **21:** 453–488.
5. Wiest, D. K., D. Wang, and D. K. Hawley. 1992. Mechanistic studies of transcription arrest at the adenovirus major late attenuation site. Comparison of purified RNA polymerase II and washed elongation complexes. *J. Biol. Chem.* **267:** 7733–7744.
6. Mok, M., A. Maderious, and S. Chen-Kiang. 1984. Premature termination by human RNA polymerase II occurs temporally in the adenovirus major late transcriptional unit. *Mol. Cell Biol.* **4:** 2031–2040.
7. Hay, N., H. Skolnik-David, and Y. Aloni. 1982. Attenuation in the control of SV40 gene expression. *Cell.* **29:** 183–193.
8. Skarnes, W. C., D. C. Tessier, and N. H. Acheson. 1988. RNA polymerases stall and/or prematurely terminate nearby both early and late promoters on polyomavirus DNA. *J. Mol. Biol.* **203:** 153–171.
9. Toohey, M. G., and K. A. Jones. 1989. In vitro formation of short RNA polymerase II transcripts that terminate within the HIV-1 and HIV-2 promoter-proximal downstream regions. *Genes Dev.* **3:** 265–282.
10. Ratnasabapathy, R., M. Sheldon, L. Johal, and N. Hernandez. 1990. The HIV-1 long terminal repeat contains an unusual element that induces the synthesis of short RNAs from various mRNA and snRNA promoters. *Genes Dev.* **4:** 2061–2074.
11. Bentley, D. L., and M. Groudine. 1986. A block to elongation is largely responsible for decreased transcription of c-myc in differentiated HL60 cells. *Nature.* **321:** 702–706.
12. Bentley, D. L., and M. Groudine. 1988. Sequence requirements for premature termination of transcription in the human c-myc gene. *Cell.* **53:** 245–256.
13. Kerppola, T. K., and C. M. Kane. 1988. Intrinsic sites of transcription termination and pausing in the c-myc gene. *Mol. Cell Biol.* **8:** 4389–4394.
14. London, L., R. G. Keene, and R. Landick. 1991. Analysis of premature termination in c-myc during transcription by RNA polymerase II in a HeLa nuclear extract. *Mol. Cell Biol.* **11:** 4599–4615.
15. Meulia, T., A. Krumm, C. Spencer, and M. Groudine. 1992. Sequences in the human c-myc P2 promoter affect the elongation and premature termination of transcripts initiated from the upstream P1 promoter. *Mol. Cell Biol.* **12:** 4590–4600.
16. Wright, S., L. F. Mirels, M. C. Calayag, and J. M. Bishop. 1991. Premature termination of transcription from the P1 promoter of the mouse c-myc gene. *Proc. Natl. Acad. Sci. USA.* **88:** 11383–11387.
17. Bender, T. P., C. B. Thompson, and W. M. Kuehl. 1987. Differential expression of c-myb mRNA in murine B lymphomas by a block to transcription elongation. *Science.* **237:** 1473–1476.
18. Watson, R. J. 1988. Expression of the c-myb and c-myc genes is regulated independently in differentiating mouse erythroleukemia cells by common processes of premature transcription arrest and increased mRNA turnover. *Mol. Cell Biol.* **8:** 3938–3942.
19. Collart, M. A., N. Tourkine, D. Belin, P. Vassalli, P. Jeanteur, and J. M. Blanchard. 1991. c-fos gene transcription in murine macrophages is modulated by a calcium-dependent block to elongation in intron 1. *Mol. Cell Biol.* **11:** 2826–2831.
20. Hipskind, R. A., and A. Nordheim. 1991. In vitro transcriptional analysis of the human c-fos proto-oncogene. *J. Biol. Chem.* **266:** 19572–19582.
21. Mechti, N., M. Piechaczyk, J. M. Blanchard, P. Jeanteur, and B. Lebleu. 1991. Sequence requirements for premature transcription arrest within the first intron of the mouse c-fos gene. *Mol. Cell Biol.* **11:** 2832–2841.
22. Chen, Z., J. W. Innis, M. H. Sun, D. A. Wright, and R. E. Kellems. 1991. Sequence requirements for transcriptional arrest in exon 1 of the human adenosine deaminase gene. *Mol. Cell Biol.* **11:** 6248–6256.
23. Chinsky, J. M., M. C. Maa, V. Ramamurthy, and R. E. Kellems. 1989. Adenosine deaminase gene expression. Tissue-dependent regulation of transcriptional elongation. *J. Biol. Chem.* **264:** 14561–14565.
24. Innis, J. W., and R. E. Kellems. 1991. A heat-labile factor promotes premature 3′ end formation in exon 1 of the murine adenosine deaminase gene in a cell-free transcription system. *Mol. Cell Biol.* **11:** 5398–5409.
25. Maa, M. C., J. M. Chinsky, V. Ramamurthy, B. D. Martin, and R. E. Kellems. 1990. Identification of transcription stop sites at the 5′ and 3′ ends of the murine adenosine deaminase gene. *J. Biol. Chem.* **265:** 12513–12519.
26. Kash, S. F., and R. E. Kellems. 1994. Control of transcription arrest in intron 1 of the murine adenosine deaminase gene. *Mol. Cell Biol.* **14:** 6198–6207.
27. Shor, J., E. Ben-Asher, and Y. Aloni. 1995. Transcription elongation of the murine ornithine decarboxylase (ODC) gene is regulated in vitro at two downstream elements by different attenuation mechanisms. *Oncogene.* **10:** 1587–1596.
28. Reines, D., D. Wells, M. J. Chamberlin, and C. M. Kane. 1987. Identification of intrinsic termination sites in vitro for RNA polymerase II within eukaryotic gene sequences. *J. Mol. Biol.* **196:** 299–312.
29. Rougvie, A. E., and J. T. Lis. 1988. The RNA polymerase II molecule at the 5′ end of the uninduced hsp70 gene of D. melanogaster is transcriptionally engaged. *Cell.* **54:** 795–804.
30. Goldring, N. B., M. Kessler, and Y. Aloni. 1992. Parameters affecting the elongation block by RNA polymerase II at the SV40 attenuator 1 in vitro. *Biochemistry.* **31:** 8369–8376.
31. Yanofsky, C. 1988. Transcription attenuation. *J. Biol. Chem.* **263:** 609–612.
32. Jackson, R. L., A. M. Gotto, O. Stein, and Y. Stein. 1975. A comparative study on the removal of cellular lipids from Landschutz ascites cells by human plasma apolipoproteins. *J. Biol. Chem.* **250:** 7204–7209.
33. Fielding, C. J., V. G. Shore, and P. E. Fielding. 1972. A protein cofactor of lecithin:cholesterol acyltransferase. *Biochem. Biophys. Res. Commun.* **46:** 1493–1498.
34. Maciejko, J. J., D. R. Holmes, B. A. Kottke, A. R. Zinsmeister, D. M. Dinh, and S. J. Mao. 1983. Apolipoprotein A-I as a marker of angiographically assessed coronary-artery disease. *N. Engl. J. Med.* **309:** 385–389.
35. Apostolopoulos, J. J., G. J. Howlett, and N. Fidge. 1987. Effects of dietary cholesterol and hypothyroidism on rat apolipoprotein mRNA metabolism. *J. Lipid Res.* **28:** 642–648.
36. Strobl, W., N. L. Gorder, Y. C. Lin-Lee, A. M. Gotto, Jr., and W. Patsch. 1990. Role of thyroid hormones in apolipoprotein A-I gene expression in rat liver. *J. Clin. Invest.* **85:** 659–667.
37. Apostolopoulos, J. J., M. J. La Scala, and G. J. Howlett. 1988. The effect of triiodothyronine on rat apolipoprotein A-I and A-IV gene transcription. *Biochem. Biophys. Res. Commun.* **154:** 997–1002.
38. Davidson, N. O., R. C. Carlos, M. J. Drewek, and T. G. Parmer. 1988. Apolipoprotein gene expression in the rat is regulated in a tissue-specific manner by thyroid hormone. *J. Lipid Res.* **29:** 1511–1522.
39. Soyal, S. M., C. Seelos, Y. C. Lin-Lee, S. Sanders, A. M. Gotto, Jr., D. L. Hachey, and W. Patsch. 1995. Thyroid hormone influences the maturation of apolipoprotein A-I messenger RNA in rat liver. *J. Biol. Chem.* **270:** 3996–4004.
40. Lin-Lee, Y. C., S. M. Soyal, A. Surguchov, S. Sanders, W. Strobl, and W. Patsch. 1995. Thyroid hormone influences conditional transcript elongation of the apolipoprotein A-I gene in rat liver. *J. Lipid Res.* **36:** 1586–1594.
41. Haddad, I. A., J. M. Ordovas, T. Fitzpatrick, and S. K. Karathanasis. 1986. Linkage, evolution, and expression of the rat apolipoprotein A-I, C-III, and A-IV genes. *J. Biol. Chem.* **261:** 13268–13277.
42. Horton, R. M., Z. L. Cai, S. N. Ho, and L. R. Pease. 1990. Gene splicing by overlap extension: tailor-made genes using the polymerase chain reaction. *Biotechniques.* **8:** 528–535.
43. Dignam, D. J., R. M. Lebovitz, and R. G. Roeder. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic. Acids Res.* **11:** 1475–1489.
44. Maxam, A. M., and W. Gilbert. 1980. Sequencing end-labeled DNA

with base-specific chemical cleavages. *Methods Enzymol.* **65:** 499–560.
45. Zuker, M., J. A. Jaeger, and D. H. Turner. 1991. A comparison of optimal and suboptimal RNA secondary structures predicted by free energy minimization with structures determined by phylogenetic comparison. *Nucleic Acids Res.* **19:** 2707–2714.
46. Bengal, E., A. Goldring, and Y. Aloni. 1989. Transcription complexes synthesizing attenuated RNA can serve as a model system for analyzing elongation factors. *J. Biol. Chem.* **264:** 18926–18932.
47. Resnekov, O., and Y. Aloni. 1989. RNA polymerase II is capable of pausing and prematurely terminating transcription at a precise location in vivo and in vitro. *Proc. Natl. Acad. Sci. USA.* **86:** 12–16.
48. Kessler, M., E. Ben-Asher, O. Resenkov, V. Hatini, E. Bengal, and Y. Aloni. 1991. A 21-base pair DNA fragment directs transcription attenuation within the simian virus 40 late leader. *J. Biol. Chem.* **266:** 13019–13027.
49. Price, D. H., A. E. Sluder, and A. L. Greenleaf. 1989. Dynamic interaction between a Drosophila transcription factor and RNA polymerase II. *Mol. Cell Biol.* **9:** 1465–1475.
50. Boshart, M., F. Weber, G. Jahn, K. Dorsch-Hasler, B. Fleckenstein, and W. Schaffner. 1985. A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. *Cell.* **41:** 521–530.
51. Aoki, T., K. S. Koch, and H. L. Leffert. 1997. Attenuation of gene expression by a trinucleotide repeat-rich tract from the terminal exon of the rat hepatic polymeric immunoglobulin receptor gene. *J. Mol. Biol.* **267:** 229–236.
52. Deuschle, U., R. A. Hipskind, and H. Bujard. 1990. RNA polymerase II transcription blocked by *Escherichia coli* lac repressor. *Science.* **248:** 480–483.
53. McStay, B., and R. H. Reeder. 1990. A DNA-binding protein is required for termination of transcription by RNA polymerase I in *Xenopus laevis*. *Mol. Cell Biol.* **10:** 2793–2800.
54. Reines, D., and J. Mote, Jr. 1993. Elongation factor SII-dependent transcription by RNA polymerase II through a sequence-specific DNA-binding protein. *Proc. Natl. Acad. Sci. USA.* **90:** 1917–1921.
55. Bengal, E., and Y. Aloni. 1989. A block of transcription elongation by RNA polymerase II at synthetic sites in vitro. *J. Biol. Chem.* **264:** 9791–9798.
56. Resnekov, O., M. Kessler, and Y. Aloni. 1989. RNA secondary structure is an integral part of the in vitro mechanism of attenuation in simian virus 40. *J. Biol. Chem.* **264:** 9953–9959.
57. Saladin, R., N. Vu-Dac, J. C. Fruchart, J. Auwerx, and B. Staels. 1996. Transcriptional induction of rat liver apolipoprotein A-I gene expression by glucocorticoids requires the glucocorticoid receptor and a labile cell-specific protein. *Eur. J. Biochem.* **239:** 451–459.
58. Vu-Dac, N., K. Schoonjans, B. Laine, J. C. Fruchart, J. Auwerx, and B. Staels. 1994. Negative regulation of the human apolipoprotein A-I promoter by fibrates can be attenuated by the interaction of the peroxisome proliferator-activated receptor with its response element. *J. Biol. Chem.* **269:** 31012–31018.
59. Berthou, L., B. Staels, I. Saldicco, K. Berthelot, J. Casey, J. C. Fruchart, P. Denefle, and D. Branellec. 1994. Opposite in vitro and in vivo regulation of hepatic apolipoprotein A-I gene expression by retinoic acid. Absence of effects on apolipoprotein A-II gene expression. *Arterioscler. Thromb.* **14:** 1657–1664.
60. Cuthbert, C., Z. Wang, X. Zhang, and S. P. Tam. 1997. Regulation of human apolipoprotein A-I gene expression by gramoxone. *J. Biol. Chem.* **272:** 14954–14960.
61. Harnish, D. C., S. Malik, and S. K. Karathanasis. 1994. Activation of apolipoprotein AI gene transcription by the liver-enriched factor HNF-3. *J. Biol. Chem.* **269:** 28220–28226.