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Competitive and Cooperative Functioning of the Anterior and Posterior Promoter Elements of an *Alu* Family Repeat

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Similar to tRNA genes and the VAI gene, the *Alu* family repeats are transcribed by RNA polymerase III and contain a split intragenic promoter. Results of our previous studies have shown that when the anterior, box A-containing promoter element (5′-Pu-Pu-Py-N-N-Pu-Pu-Py-G-G-3′ in which Pu is any purine, Py is any pyrimidine, and N is any nucleotide) of a human *Alu* family repeat is deleted, the remaining box B-containing promoter element (5′-G-A/T-T-C-Pu-A-N-N-C-3′) is still capable of directing weak transcriptional initiation at appproximately 70 base pairs (bp) upstream from the box B sequence. This is different from the tRNA genes in which the box A-containing promoter element plays the major role in the positioning of the transcriptional initiation site(s). To account for this difference, we first carried out competition experiments in which we show that the posterior element of the *Alu* repeat competes with the VAI gene effectively for the transcription factor C in HeLa cell extracts. We then constructed a series of contraction and expansion mutants of the *Alu* repeat promoter in which the spacing between boxes A and B was systematically varied by molecular cloning. In vitro transcription of these clones in HeLa cell extracts was analyzed by RNA gel electrophoresis and primer extension mapping. We show that when the box A and box B promoter sequences are separated by 47 to 298 bp, the transcriptional initiation sites remain 4 to 5 bp upstream from box A. However, this positioning function by the box A-containing promoter element was lost when the spacing was shortened to only 26 bp or increased to longer than 600 bp. Instead, transcriptional initiation occurred approximately 70 bp upstream from box B, similar to that in the clones containing only the box B promoter element. All the mutant clones were transcribed less efficiently than was the wild type. An increase in the distance between boxes A and B also activated a second box A-like element within the *Alu* family repeat. We compare these results with the results of tRNA gene studies. We also discuss this comparison in terms of the positioning function of the split class III promoter elements and the evolutionary conservation of the spacing between the two promoter elements for optimum transcriptional efficiency.

Eucaryotic genes are transcribed by three types of RNA polymerases: type I synthesizes the large rRNA; type II synthesizes protein-coding mRNA; type III synthesizes tRNA, 5S RNA, small viral RNA, and *Alu*-like repetitive sequences. While the class I and II genes contain their promoter sequences near and upstream from the RNA initiation sites (see references 12 and 30 and references therein), the promoters of polymerase III-dependent genes are located within the transcribed regions (for a review, see reference 6).

Ciliberto et al. (7) have divided the RNA polymerase III-dependent genes into two classes. The first class consists of the tRNA, VAI RNA, and *Alu*-like repeat genes. The second type is the 5S RNA gene. All of the transcription units of the first class contain two split intragenic promoter regions separated by approximately 30 to 60 base pairs (bp) (3, 8, 10, 11, 14–16, 18, 20, 25, 32, 33, 35, 38, 46). The anterior promoter region contains a highly conserved DNA sequence 5′-Pu-Pu-Py-N-N-Pu-Pu-Py-G-G-3′ (box A), in which Pu is any purine, Py is any pyrimidine, and N is any nucleotide, located 5 to 18 bp downstream from the 5′ end of the tRNA, adenovirus VAI, and *Alu*-like repeat genes. The posterior promoter region contains the conserved sequence 5′-G-A/T-T-C-Pu-A-N-N-C-3′ (box B), which is located approximately 30 bp (in VAI and tRNA genes) to 60 bp (in *Alu*-type repeats) downstream from the box A sequence (7, 18) (Fig. 1A). The 5S RNA gene differs from the class of polymerase III-dependent genes described above in that it has a contiguous block of intragenic promoter from bases 50 to 83 (4, 37). The first 11 bp of the 5S RNA promoter, however, is homologous to the box A promoter sequence of tRNA, VAI, and *Alu*-like repeat genes (7).

Multiple cellular factors are required for the transcriptional initiation of the tRNA, VAI, and 5S RNA genes (13, 17, 21, 26–28, 34, 43, 44, 48). A common factor, termed factor C by Lassar et al. (28), specifically binds to the box B promoter sequence of the tRNA and VAI genes. This interaction is necessary and sufficient for the formation of a stable transcription complex of the VAI gene, while an additional factor, factor B, is required for tRNA genes (28). Results of recent studies have shown that the transcription of cellular or transfected class III genes can be enhanced by viral products, presumably because of the increased activity of transcription factor(s) in the transformed cells (2, 19, 24, 41).

We have previously studied the organization and function of the intragenic promoter of a human *Alu* family repeat located downstream from the α1-globin gene (35). Transcriptional analysis of 5′ and 3′ deletion clones of this repeat in vitro identified two split intragenic promoter elements: the directing element (bases 70 to 86), containing a box B-like sequence, and the enhancing element (bases 4 to 37), containing a box A-like sequence (35) (Fig. 1B). This bipartite structure of the *Alu* repeat promoter is consistent with the observation of Paolella et al. (33).

There is one interesting, but puzzling, feature of the functional organization of the *Alu* promoter elements in comparison with other class III genes. Similar to the VAI

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and tRNA genes, deletion of the box B-like sequence within the *Alu* repeat completely abolishes the transcriptional activity. However, although deletion of the box A-like sequence of the *Alu* repeat reduces the efficiency of transcription by 10- to 20-fold, the remaining box B-containing promoter element is sufficient to direct initiation of transcription at approximately 70 bp upstream (35). This is in contrast to the results obtained from other class III genes, in which the box B-containing promoter element alone is not capable of initiating transcription in a homologous extract (for a review, see reference 6), although point mutations in the box A-containing promoter element of a *Drosophila* $tRNA^{Arg}$ gene does decrease its transcriptional efficiency (42). Furthermore, expansion of the spacing between the box A and box B promoter sequences of tRNA genes results in transcriptional initiation at 10 to 18 bp upstream from the box A sequence (8, 18, 38). This suggests that the box A, but not the box B, promoter sequence plays the positioning role in the transcriptional initiation of tRNA genes. Because our approach (35) is different from that used by other workers, i.e., transcription of BAL 31 deletion clones instead of expansion mutants, the difference between the *Alu* repeats and tRNA genes could not be easily resolved.

To get a better understanding of the mechanism(s) of the positioning of initiation sites by the class III promoter elements, we constructed a series of expansion and contraction mutants of the 3′-α1-globin *Alu* repeat (40), with the distance between the box A and box B sequences varying from 26 to 298 bp. We report here the transcriptional analysis of these constructs in homologous HeLa cell extracts and discuss the positioning function of the class III promoter elements in terms of these results.

MATERIALS AND METHODS

Construction of parental plasmid clones. Three parental plasmid clones, pAlu-(+59/+205), pi-Alu-(78), and pi-Alu-(96), were constructed as follows. A 146-bp *Sau*3A fragment containing bases 59 to 205 of the 3′-α1-globin *Alu* repeat (Fig. 1B) was blunt-ended with Klenow enzyme (Biotech Laboratories), and *Eco*RI linkers (Collaborative Research, Inc., Waltham, Mass.) were ligated to its ends. Insertions of this fragment into the *Eco*RI site of pBR322 resulted in the clone pAlu-(+59/+205). Plasmid pi-Alu-(78) was constructed by cloning a 74-bp *Hinc*II-*Bst*NI fragment (−30 to 44) of the *Alu* repeat (see Fig. 3A) into the *Hin*dIII site of pBR322 after a reaction to make it blunt-ended and the addition of *Hin*dIII linkers. Plasmid pi-Alu-(96) was constructed by cloning the 92-bp *Hinc*II-*Sau*3A fragment (−30 to 62) of the *Alu* repeat (see Fig. 3A) into the *Hin*dIII site of pBR322 after blunt-ended reaction and addition of *Hin*dIII linkers. Note that the numbers in the parentheses of pi-Alu-(78) and pi-Alu-(96) represent the distances (in base pairs) between box A and B (see Fig. 7). (See Fig. 3A for maps of these three plasmid clones.) They were used to construct the contraction, expansion, and inversion mutants of the Alu repeat promoter (see below).

Construction of contraction mutant clones. Plasmid pi-Alu-(96) was linearized with restriction enzyme *Cla*I (see Fig. 3A) and digested with exonuclease BAL 31 (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) to different extents. The digested molecules were made blunt-ended, recircularized by ligation, and transformed into *Escherichia coli* RRI. The exact extent of deletions was determined by DNA sequencing (29). The maps of the contraction clones used for transcriptional analysis are shown together with the expansion clones in Fig. 4.

Construction of expansion mutant clones. The spacing between the enhancing and directing promoter elements of the *Alu* repeat was increased by inserting DNA fragments of known sequences into the *Cla*I site of plasmid pi-Alu-(78) or pi-Alu-(96). The DNA fragments used were *Hpa*II restriction fragments, derived from the human α2-αl-globin intergenic region (22, 31), with lengths of 38, 45, 71, 198, and 503 bp. The maps of these expansion mutant clones are shown together with the contraction clones in Fig. 4.

Construction of inversion mutant and shotgun clones. To construct *Alu* mutants with reversed orientation of the box A sequence, the 92-bp *Hin*dIII fragment of plasmid pi-Alu-(96) (see above) was cleaved with *Hae*III, and the resulting 60-bp *Hae*III-*Hin*dIII fragment containing the box A promoter sequence was purified by gel elution. The fragment was made blunt-ended, ligated to *Hin*dIII linkers, and cloned into the *Hin*dIII site of plasmid pAlu-(+59/+205) (see above) in both orientations, resulting in clones pi-Alu-(96)-I and pri-Alu-(53) (see Fig. 8A). Note that pi-Alu-(96)-I was essentially the same as pi-Alu-(96), except that the 5′-flanking human DNA in pi-Alu-(96) was substituted by the pBR322 sequence in pi-Alu(96)-I.

For construction of shotgun clones, the plasmid pAlu-(+59/+205) (see Fig. 3A) was randomly linearized by partial cleavage with restriction enzyme *Sau*3A. Full-length fragments were purified by agarose gel elution and used as cloning vectors. The 60-bp *Hae*III-*Sau*3A fragment of 3′ α1-globin-*Alu* repeat, which consists of DNA sequence from bases 2 to 62, including the enhancing element (35), was randomly cloned into the *Sau*3A sites of this collection of linear pAlu-(+59/+205) molecules after the addition of *Bam*HI linkers to its ends. Clones which varied the placement (600 to 3,000 bp) of the enhancing element, but in the correct orientation, from the directing element were then selected for transcriptional analysis in vitro.

Transcription in vitro and RNA mapping. HeLa S-100 extracts were prepared (47, 49) and used to transcribe plasmid DNA as described previously (35). ^{32}P-labeled RNAs synthesized in the transcription mixtures were analyzed by gel electrophoresis in 7 M urea–6% polyacrylamide gels and by autoradiography. Primer extension analysis with ^{32}P-end-labeled, single-stranded DNA probes was performed as described previously (23, 45).

Transcriptional competition. Transcriptional competition experiments were performed by the method of Fuhrman et al. (17) with some modifications. Different amounts of a plasmid DNA (competitor DNA template) were transcribed at 30°C in HeLa S-100 extracts under the conditions described by Wu (49). After 10 min, a constant amount of a reference DNA template was added to each preincubation mixture. The total quantity of DNA in each mixture was brought up to 3.5 μg with pBR322 DNA. After further incubation at 30°C for 1 h, the ^{32}P-labeled RNAs were purified and analyzed by gel electrophoresis and autoradiography (see Fig. 2).

RESULTS

Transcription of plasmid clone pRBα1 containing an *Alu* repeat in a HeLa S-100 extract resulted in the synthesis of three RNA species: RNA-a (404 to 408 nucleotides [nt]), RNA-b (252 to 255 nt), and RNA-c (173 to 174 nt). All of these RNA species were initiated by RNA polymerase III at the first base of the repeat sequence (23, 35). The major

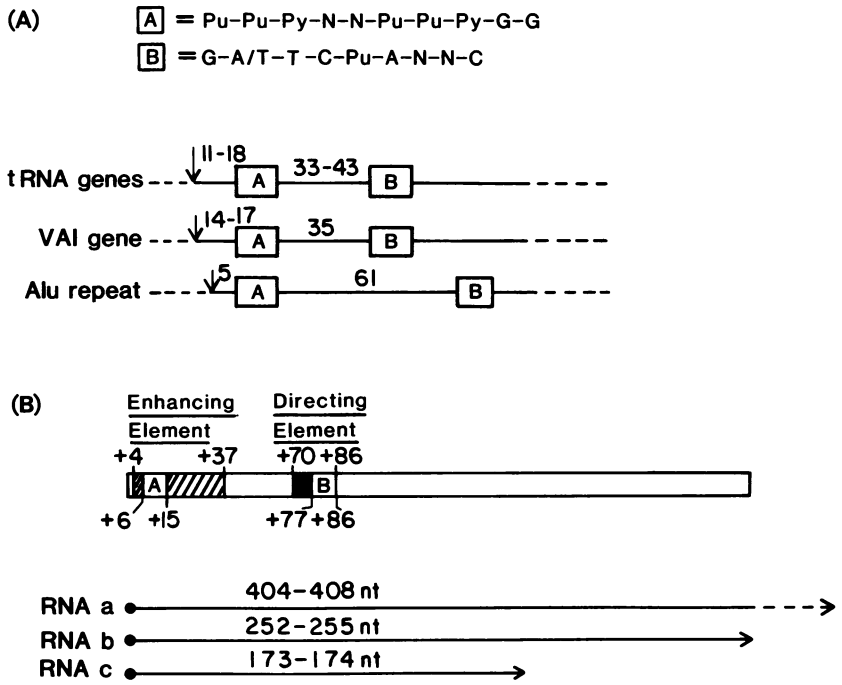

FIG. 1. (A) Alignment of the conserved promoter sequences, box A and box B, of tRNA, VAI, and *Alu* family repeat genes. The DNA sequences of boxes A and B, as shown on the top two lines, are derived from those of Ciliberto et al. (7). The arrows indicate the transcriptional initiation sites upstream from box A. The numbers indicate the distances (in bases) between the initiation sites and the 5′ border of box A, as well as the distances between the 3′ border of box A and 5′ border of box B. Pu, purine; Py, pyrimidine; N, any base. (B) Transcription map and promoter organization of the 3′-α1-globin·*Alu* repeat. The three RNAs initiated from the first base of the *Alu* repeat have been mapped by Hess et al. (23). RNA-a is a readthrough transcript. The enhancing element (bases 4 to 37; hatched box) and the directing element (bases 70 to 86; solid box) of the *Alu* repeat have been mapped by Perez-Stable et al. [35]; see text). Box A (bases 6 to 15) and box B (bases 77 to 86) sequences are contained within the enhancing element and the directing element, respectively.

transcript, RNA-b, terminated near the 3′ end of the repeat at a new form of polymerase III terminator. The RNA-c terminated at two thymine residues (nt 173 and 174) within the *Alu* repeat. RNA-a was a readthrough transcript terminating in the pBR322 sector (23) (Fig. 1B).

The posterior, directing element of the *Alu* repeat promoter competes with VAI gene for transcription factor(s) in vitro. Results of in vitro transcription studies of subclones and BAL 31 deletion clones of the 3′ α1-globin *Alu* repeat have identified two functionally distinct promoter elements located within the gene (35). The anterior element (nt 4 to 37) by itself cannot initiate transcription. Deletion of this element from the wild-type *Alu* sequence, however, reduces the efficiency of transcription by 10- to 20-fold. The posterior element (mapped at nt 70 to 82 by Perez-Stable et al. [35]) alone is sufficient to direct initiation at approximately 70 bp upstream from its 5′ boundary. The anterior and posterior elements were termed enhancing and directing elements, respectively (35). Results of more recent experiments have established the essential role of the cytosine residue at position 86. When this base was mutated to adenine via subcloning, the transcriptional activity of the *Alu* repeat in vitro was completely abolished (C. Perez-Stable, T. M. Ayres, and C.-K. J. Shen, unpublished data). This cytosine was a highly conserved nucleotide also found at the 3′ end of the box B sequence in the tRNA and VAI genes and is probably involved in the interaction between a transcriptional factor and the class III promoter (see reference 5 and references therein). The border of the directing element is thus defined as from bases 70 to 86, which includes the entire box B sequence (Fig. 1B).

We carried out experiments to test whether transcription of the *Alu* repeat involves the interaction of its promoter elements with a factor(s) that is also utilized by the VAI and tRNA genes in vitro. The wild-type *Alu* repeat was able to compete effectively with the VAI gene for the factor(s) in the transcription extract (Fig. 2A). Thus, preincubation of the VAI gene at a concentration of 10^{-3} μM (Fig. 2A, lane 5) effectively prevents the transcriptional initiation of the *Alu* repeat subsequently added at a concentration of 7.2×10^{-3} μM. Conversely, preincubation of 7.2×10^{-3} μM of the *Alu* repeat in the transcription extract (Fig. 2A, lane 11) abolishes the transcription of VAI gene at a concentration of 2×10^{-3} μM. These data suggest that the promoters of the *Alu* repeat and VAI genes interact with the same factor(s) present in the extract with comparable strengths.

A 5′ deletion clone p5d-Alu-(−219/+69), which contains only the directing element, is transcribed at a 5 to 10% efficiency of that of the wild type Alu (35). However, it is able to compete with the VAI gene for transcription factor(s), although not as efficiently as the wild-type *Alu* repeat (Fig. 2B). On the contrary, the 3′ deletion clone p3d-(+63/+551), which contains only the anterior enhancing element of the *Alu* promoter, is not capable of competing with the VAI gene (Fig. 2B), a result which is similar to those observed in the competition study of tRNA and 5S RNA genes (17, 39, 48). These results suggest that the major binding site of factor(s) to the *Alu* repeat is also contained within the posterior directing element, similar to the VAI and tRNA genes.

Altering spacing between the enhancing and directing elements enhances the initiation immediately upstream from a

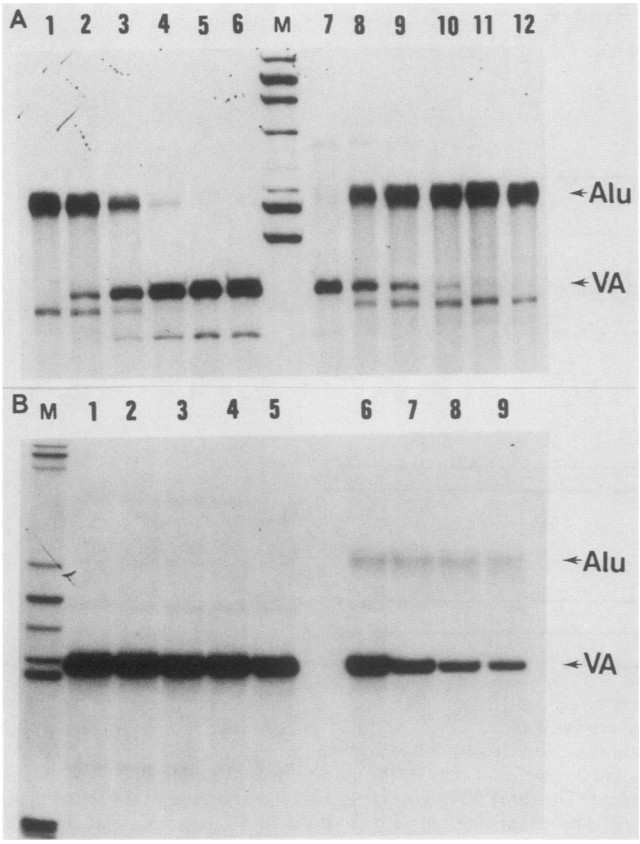

FIG. 2. Competition of *Alu* repeat promoter with the VAI gene for transcription factor(s) in HeLa S-100 extracts. (A) Competiton between the wild-type *Alu* repeat and the VAI gene. In lanes 1 to 6, 2 μg of plasmid pRBα1 containing the 3′-α1-globin *Alu* repeat (40) was used as the reference DNA, and a plasmid (pVAI) containing an intact copy of VAI gene was used as the competitor DNA. In lanes 7 to 12, 0.5 μg of the pVAI was used as the reference template, while pRBα1 was the competitor DNA. The quantities (in micrograms) of the competitor DNA used were as follows: lane 1, 0; lane 2, 0.01; lane 3, 0.05; lane 4, 0.1; lane 5, 0.25; lane 6, 0.5; lane 7, 0; lane 8, 0.25; lane 9, 0.5; lane 10, 1; lane 11, 2; lane 12, 3. M, Single-stranded DNA marker. (B) Competition of *Alu* repeat mutants with VAI gene. In all lanes, 0.5 μg of pVAI was used as the reference DNA template. The plasmid p3d-(+63/+551), which lacks the posterior promoter element (see text [35]), was used as the competitor DNA in lanes 2 to 5. The plasmid p5d-(−219/+69), which lacks the anterior element (see text [35]), was used as the competitor DNA in lanes 6 to 9. The amounts (in micrograms) of competitor DNA used were as follows: lane 1, 0; lanes 2 and 6, 0.5; lanes 3 and 7, 1; lanes 4 and 8, 2; lanes 5 and 9, 3. M, Single-stranded DNA marker.

second box A-like promoter element within the *Alu* repeat sequence. Several recombinant clones were made as the parental plasmids for subsequent construction of expansion, contraction, and inversion mutants of the *Alu* repeat promoter. A 146-bp *Sau*3A DNA fragment containing the directing element, and thus the box B sequence, was cloned into the *Eco*RI site of pBR322 with *Eco*RI linkers (see above). This clone was designated pAlu-(+59/+205) (Fig. 3A). Two different restriction fragments, each containing the enhancing element, were then cloned into the *Hin*dIII site of pAlu-(+59/+205), resulting in the plasmids pi-Alu-(78) and pi-Alu-(96) (Fig. 3A). The relative directions of the box A and box B promoter sequences in these two plasmids were the same as that in the wild type. However, the spacings between the two boxes in these plasmids were 78 and 96 bp, respectively, instead of 61 bp as in the wild-type *Alu* repeat (see also Fig. 7).

The transcriptional patterns of these three plasmids were compared with the wild-type *Alu* repeat (Fig. 3B). As mentioned above, the wild-type *Alu* repeat directs the synthesis of RNA-a, -b, and -c (Fig. 3B, lane 1). The major transcript, RNA-b, terminates at the oligo(dA) stretch near the 3′ end of the *Alu* repeat. RNA-a and -c are readthrough and premature transcripts, respectively, both terminating at the conventional, typical RNA polymerase III terminator (3 or more thymines). Plasmid pAlu-(+59/+205) was transcribed at only a 10% efficiency of that of the wild type (Fig. 3B, lane 2). This is consistent with the previous observation (35) that the enhancing element is required for a high efficiency of transcription in vitro. However, if the enhancing element was placed back upstream from the directing element, as in plasmids pi-Alu-(78) and pi-Alu-(96), the overall efficiency of transcription was greatly increased (Fig. 3B, lanes 3 and 4). The transcripts of plasmids pAlu-(+59/+205), pi-Alu-(78), and pi-Alu-(96) terminated either at bases 173 to 174 of the *Alu* sequence or at a cluster of five thymines in the pBR322 vector (23) (Fig. 3A).

The initiation sites of these transcripts were mapped by the primer extension technique. A single-stranded *Bst*NI-*Sau*3A fragment, labeled with ^{32}P at the *Sau*3A end (Fig. 3A), was hybridized with gel-purified RNAs transcribed from pAlu-(+59/+205), pi-Alu-(78), or pi-Alu-(96) and extended with reverse transcriptase. The extension products were analyzed on a denaturing polyacrylamide gel (Fig. 3C). It was found that the initiation sites in pAlu-(+59/+205) were located at 75 and 86 bp upstream from the directing element. Initiation in pi-Alu-(78) was at the normal wild-type site, namely the 5′-most base of the *Alu* repeat, which in this clone was 94 bp upstream from the directing element. These sites are indicated by arrows in Fig. 3A.

An increase in the spacing between boxes A and B from 78 to 96 bp enhanced the transcriptional initiation from a site located within the *Alu* sequence. There were four transcripts with approximate lengths of 284, 244, 210, and 170 nt synthesized from pi-Alu-(96) in the HeLa S-100 extract (Fig. 3B, lane 4). Primer extension analysis (Fig. 3C) indicated that while the 284-nt (Fig. 3C, lane 4) and 210-nt RNA (data not shown) species were initiated near the 5′ end of the enhancing element, the other two RNAs (Fig. 3A and B) were the result of initiation at base 44 within the *Alu* sequence. This initiation site was 7 bp upstream from a second box A-like sequence (box A′, bases 51 to 59) (see Fig. 7), which has been noted previously and thought to be part of the *Alu* repeat promoter (40). Interestingly, in plasmid pi-Alu-(96), initiation from this site was 3 times more prominent than that initiated upstream from the posterior box A-like promoter sequence (Fig. 3B, lane 4). Transcriptional initiation immediately upstream from this box A′ sequence also occurred efficiently on plasmid pi-Alu-(88), the interbox spacing of which was 88 bp (see the transcripts denoted with an asterisk in Fig. 5, lane 8). It could also be observed, although at very low levels, in the other clones (e.g., Fig. 5, lanes 4 to 6).

Transcriptional analysis of contraction and expansion promoter mutants. The experiments described above show that when the spacing between the box A and B promoter elements was changed from 61 to 78 or 96 bp, the transcriptional initiation still occurred near the 5′ boundary of box A or the enhancing element. In other words, the positioning of

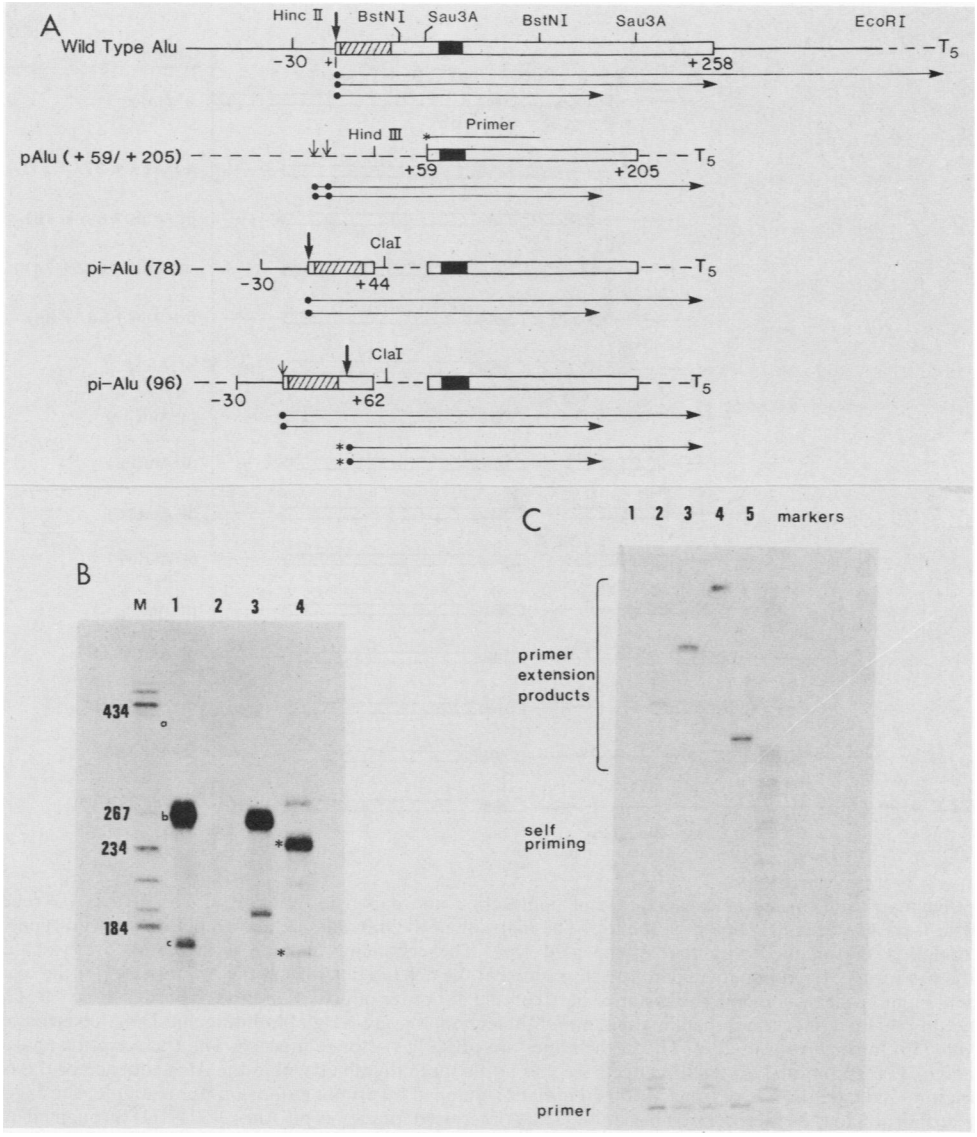

FIG. 3. Transcriptional analysis in vitro of wild-type and three mutant *Alu* repeat sequences. (A) Transcriptional map and promoter arrangement of the wild-type *Alu* (3′-α1-globin *Alu*) in plasmid pRBα1 and three mutant *Alu* repeat sequences in pAlu-(+59/+205), pi-Alu-(78), and pi-Alu-(96). The construction of these plasmids is described in the text. The *Alu* sequence is represented by the open bar, the enhancing element (bases 4 to 37) as the hatched box, and the directing element (70 to 86) as the black box. Flanking human DNA is represented by the thin line; pBR322 vector sequence is represented by the dashed line. The numbers (in bases) refer to the positions of *Alu* repeat sequences relative to the initiation site (base 1) of the wild-type *Alu* repeat. The RNA transcripts generated in vitro were analyzed by gel electrophoresis (Fig. 3B) and primer extension analysis (Fig. 3C). The arrows indicate the sites of transcriptional initiation in each clone, and their sizes are in approximate proportion to the efficiency of initiation. The two transcripts of clone pi-Alu-(96) with asterisks (see Fig. 3B) were initiated upstream from a second box A-like sequence between the enhancing and directing elements (see text). Termination of transcription of all three mutants occurred at two places: a minor one at bases 173 and 174 which was similar to the wild-type *Alu* repeat and a major one at a cluster of five thymine residues (T_5) in the pBR322 vector. The primer, a single-stranded *Bst*NI-*Sau*3A fragment labeled at the *Sau*3A end, was used to map the initiation sites and is shown on top of the map of pAlu-(+59/+205). (B) Transcription pattern of pRBα1, pAlu-(+59/+205), pi-Alu-(78), and pi-Alu-(96) in vitro. The RNAs synthesized in the HeLa S-100 extracts were purified and analyzed on denaturing gels. Markers (M) used were single-stranded *Hae*III-pBR322 fragments; lane 1, pRBα1; lane 2, pAlu-(+59/+205); lane 3, pi-Alu-(78); lane 4, pi-Alu-(96). The transcripts denoted by an asterisk in lane 4 are initiated from the second box A-like element in the *Alu* repeat (see text). Numbers to the left of the gel are in bases. (C) Primer extension analysis of transcripts from the gel shown in panel B. RNAs were eluted from the gel shown in panel B and analyzed by primer extension with an 82-nt *Bst*NI-*Sau*3A fragment as the primer. The RNA samples used were as follows: lane 1, no RNA; lane 2, 260-nt RNA from pAlu-(+59/+205); lane 3, 260-nt RNA from pi-Alu-(78); lane 4, 284-nt RNA from pi-Alu-(96); lane 5, 244-nt RNA from pi-Alu-(96). DNA sequencing markers were coelectrophoresed to measure the lengths of the extension products.

the RNA 5′ end(s) seems to be directed by the enhancing element. To test further the generality of this conclusion, a series of contraction and expansion mutants was derived from plasmids pi-Alu-(96) and pi-Alu-(78) by molecular cloning, as described above. The interbox spacing of these mutants range from 26 to 298 bp (Fig. 4).

The transcriptional patterns of these promoter mutants were compared to the wild-type *Alu* repeat (Fig. 5). The

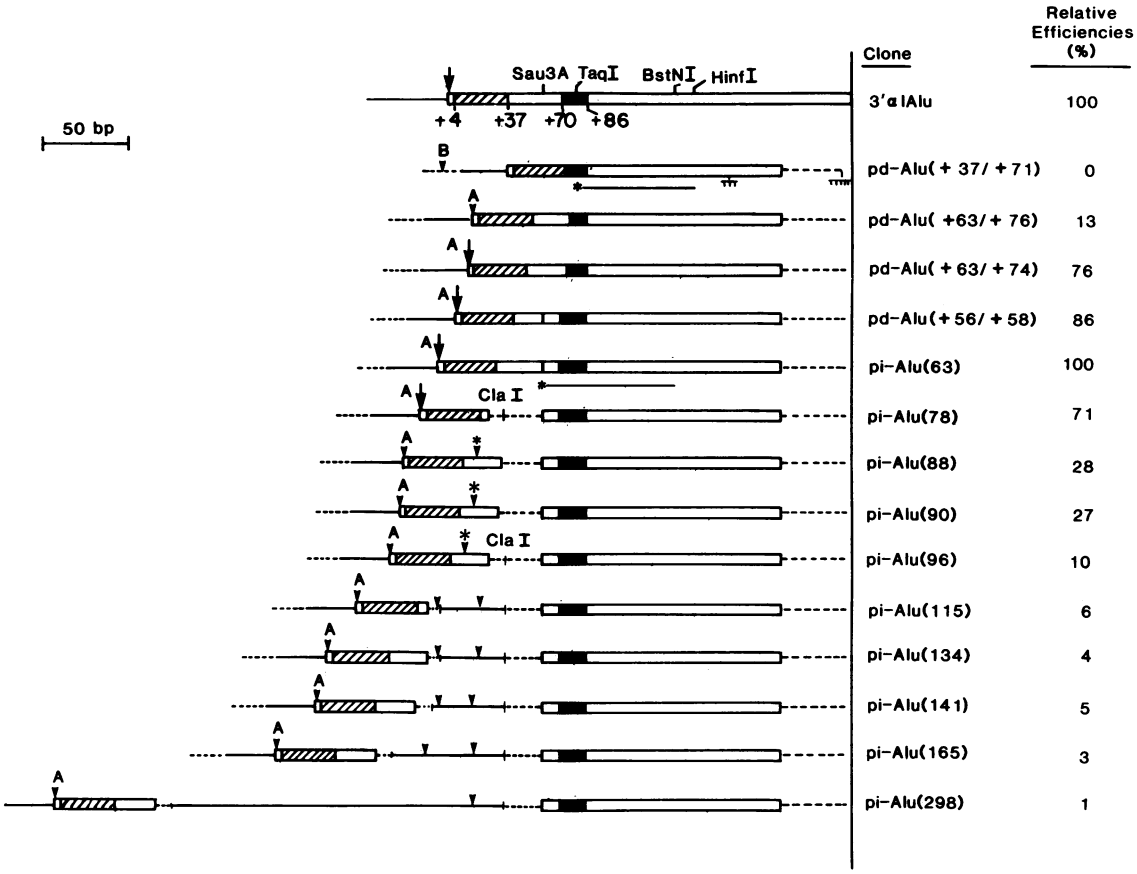

FIG. 4. Transcription maps and promoter organization of contraction and expansion mutant clones of the *Alu* repeat. The promoter organization of the wild-type *Alu* repeat is shown on the top. The maps of all the mutants are shown below the wild type with their directing promoter elements (black boxes) aligned with that of the wild type. The enhancing element is indicated by the hatched box. For the contraction clones (the p-d series), the numbers in parentheses represent the deleted portions of the *Alu* repeat. For the expansion clones (the p-i series), the numbers in parentheses indicate the distance between the 3′ border of box A and the 5′ border of box B. Open bars represent the *Alu* sequence; the thin line is either the 5′-flanking human DNA sequence or α2-α1-globin intergenic DNA inserted between box A and box B of clones pi-Alu-(115) through pi-Alu-(298). The dashed lines are pBR322 vector sequences. The transcription analysis of these clones are shown in Fig. 5 and 6. Transcriptional termination occurs either at the three thymines within the *Alu* sequence or the five thymines in the pBR322 vector. The arrows indicate the site of transcriptional initiation mapped by primer extension (for example, see Fig. 6), with their sizes being in approximate proportion to the efficiencies of initiation. The primer used for clones pd-Alu-(+37/+71) through pd-Alu-(+56/+58) was a single-stranded *Taq*I-*Hin*fI fragment labeled at the *Taq*I end; the primer used for clones pi-Alu-(63) through pi-Alu-(298) was a single-stranded *Sau*3A-*Bst*NI fragment labeled at the *Sau*3A end. In the column of relative efficiencies, only those of the initiation upstream from the box A are included. The wild-type efficiency was taken as 100%. The nucleotide sequence surrounding the initiation sites are shown in Fig. 7.

transcriptional efficiency was reduced 10-fold when box A and box B sequences were 26 or 47 bp apart, as in deletion clones pd-Alu-(+37/+71) and pd-Alu-(+63/+76), respectively (Fig. 5, lanes 2 and 3). Transcriptional efficiency was restored to 70 to 100% of the wild-type level when the spacing was between 49 and 78 bp (compare lanes 4 to 7 with lane 3 in Fig. 5). When the two boxes were separated by 88, 90, or 96 bp, the overall efficiency was again reduced by a factor of 2 to 3 (Fig. 5, lanes 8 to 10). Further reduction of the transcriptional efficiency was observed for clones with the interbox spacings varying between 115 and 298 bp (Fig. 5, lanes 11 to 15). These clones were transcribed at approximately 10 to 20% of the wild-type level.

The size increase of the two major classes of RNAs (indicated by arrows and denoted as A_1 and A_2, respectively, Fig. 5) transcribed from the mutant clones pd-Alu-(+63/+76) through pi-Alu-(298) (Fig. 5, lanes 3 to 15) correlated well with the increase of the spacings between the box A- and box B-containing promoter elements. This was expected if the two classes of RNAs were both generated by initiation at a relatively fixed distance upstream from the box A sequence and by termination at bases 173 to 174 within the *Alu* repeat (A_2 series of RNAs, Fig. 5), or at the thymine cluster in the pBR322 vector (A_1 series of RNAs, Fig. 5). Indeed, primer extension analysis of these transcripts eluted from the gel demonstrated that they were all initiated 4 to 5 bp upstream from the box A sequence, at the first or the second base of the *Alu* repeat (Fig. 6). The mapping data of the A_1 series of RNAs are shown in Fig. 6 (lanes 2 to 13), and the initiation sites mapped are indicated by arrows in Fig. 4 and 7.

The transcription of the clone pd-Alu-(+37/+71) was interesting. The box A and box B promoter sequences in this clone are separated by only 26 bp (Fig. 4 and 7). Unlike clones pd-Alu-(+63/+76), pd-Alu-(+63/+74), and pd-Alu-(+56/+58) described above, in which the spacings between

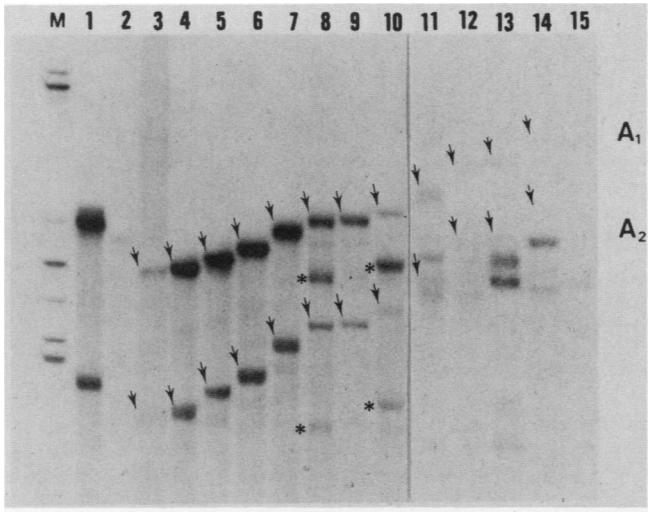

FIG. 5. Transcriptional patterns of *Alu* contraction and expansion clones. The promoter mutant clones of Fig. 4 were transcribed in HeLa S-100 extracts, and their RNA products were analyzed by gel electrophoresis as described in the text. The A_1 and A_2 series of bands marked with arrows on the autoradiograph correspond to RNAs initiated upstream from the anterior box A promoter element and terminate at the five or three thymines (Fig. 4 and text), respectively. The asterisks in lanes 8 and 10 denote RNAs initiated upstream from the second box A sequence within the *Alu* repeat (Fig. 3 and text). The faint bands in lanes 11 to 15 are shown more clearly on a longer exposure of the gel, although pi-Alu-(298) A_1 and A_2 RNAs (lane 15) are too faint to be seen. Lane 1, wild-type Alu; lane 2, pd-Alu-(+37/+71); lane 3, pd-Alu-(+63/+76); lane 4, pd-Alu-(+63/+74); lane 5, pd-Alu-(+56/+58); lane 6, pi-Alu-(63); lane 7, pi-Alu-(78); lane 8, pi-Alu-(88); lane 9, pi-Alu-(90); lane 10, pi-Alu-(96); lane 11, pi-Alu-(115); lane 12, pi-Alu-(134); lane 13, pi-Alu-(141); lane 14, pi-Alu-(165); lane 15, pi-Alu-(298). Markers (M) are single-stranded *Hae*III-pBR322 fragments.

boxes A and B were also shorter than that in the wild type, the transcription of pd-Alu-(+37/+71) was not initiated immediately upstream from the box A sequence. Instead, similar to the wild-type *Alu* repeat, it was initiated 74 bp upstream from the box B sequence, as determined by primer extension analysis (Fig. 6, lane 2). The positions of the initiation sites of pd-Alu-(+37/+71) seem to be determined by the directing element, despite the existence of the box A sequence within the transcription unit.

In addition to the initiation events described above, several clones contained other transcriptional initiation sites which gave rise to a more heterogeneous population of RNAs. The 5′ ends of many of these RNAs have also been mapped by primer extension (data not shown). The RNAs denoted with an asterisk in Fig. 5, lanes 8 and 10, for example, are the result of initiation upstream from the box A′-like sequence at bases 51 to 59, as described above in detail for the clones pi-Alu-(96) (Fig. 3). There were also RNAs initiated from the human DNA inserts cloned in between the two promoter elements of clones pi-Alu-(115), pi-Alu-(134), pi-Alu-(141), pi-Alu-(165), and pi-Alu-(298). These initiation sites are indicated by arrows in Fig. 4 and 7. Most of them were located immediately upstream from sequences homologous to the box A promoter sequence (Fig. 7).

Transcriptional analysis of a clone with reversed orientation of the enhancing element. The enhancing element containing box A is essential for the efficient transcription of the *Alu* repeat in vitro (35). Whether the enhancing element functions in the opposite orientation, similar to that of the eucaryotic polymerase II-dependent enhancer (1), is still an open question. To answer this, we carried out the following experiment.

A *Hae*III-*Sau*3A fragment spanning bases 2 to 62 of the wild-type *Alu* repeat was cloned in either orientation upstream from the directing element (see above; Fig. 8A). The clone with the wild-type orientation of the enhancing element, pi-Alu-(96)-I, was almost identical to clone pi-Alu-(96) (Fig. 3A), except that the 5′-flanking human DNA region was substituted with a pBR322 sequence. Similar to pi-Alu-(96), this clone was transcribed efficiently in vitro (Fig. 8B, lane 2) with the initiation site mapped at bases 1 and 44 (Fig. 8A and C). On the contrary, the reverse orientation clone pri-Alu-(53) (Fig. 8A) was transcribed weakly (Fig. 8B, lane 3) from a number of initiation sites, some of which were close to box A-like sequences, while others seemed to be positioned by the directing element (Fig. 8C).

DISCUSSION

Two unique characteristics distinguish the promoters of class III genes from those of the class II genes. First, the promoters of the class III genes are located within the transcribed regions. Second, each RNA polymerase III-dependent promoter is split into two elements. The promoter organization of tRNA genes previously have been shown to be plastic. That is, the distance between the two split elements can be varied, within a certain range, without affecting the accuracy of transcriptional initiation. In this report we provide a detailed study of the transcriptional regulation in vitro of contraction and expansion mutants of an *Alu* family repeat. By doing these experiments, we have attempted to answer three questions. (i) Which promoter element, the posterior or the anterior one, plays a major role in positioning the transcriptional initiation site? (ii) What is the effect of changing the interpromoter element distance on the transcription of the *Alu* family repeat in comparison with that in tRNA genes? (iii) What is the requirement for transcription factor(s) of the *Alu* repeat in comparison with other class III genes?

Positioning of the transcriptional initiation site. Results of our previous studies with BAL 31 deletion clones (35) have shown that the posterior promoter element of the *Alu* repeat is able to direct transcription by itself. Furthermore, the distance between the transcriptional initiation site(s) and the posterior promoter element is approximately the same as that in the wild-type *Alu* repeat (35). This is not the case for tRNA genes or the VAI gene, in which a cooperative interaction between the two promoter elements is required for accurate initiation to occur and the initiation site(s) remain a fixed distance upstream from the anterior promoter element (8, 18, 20, 38).

By using an approach similar to that used for tRNA genes (8, 38), we analyzed the transcriptional patterns of a number of *Alu* repeat mutants in which interpromoter element distances are different from those of the wild type (Fig. 5). Our RNA mapping results (Fig. 7) indicate that when the two *Alu* promoter elements are present in *cis* and separated by more than 26 bp, transcriptional initiation occurs at 4 to 5 bp upstream from the box A sequence. This initiation event occurs even when boxes A and B are separated by as far as 298 bp [clone pi-Alu-(298), Fig. 4 to 7]. *Alu* repeat mutants with interbox distances that vary from 600 bp to longer than

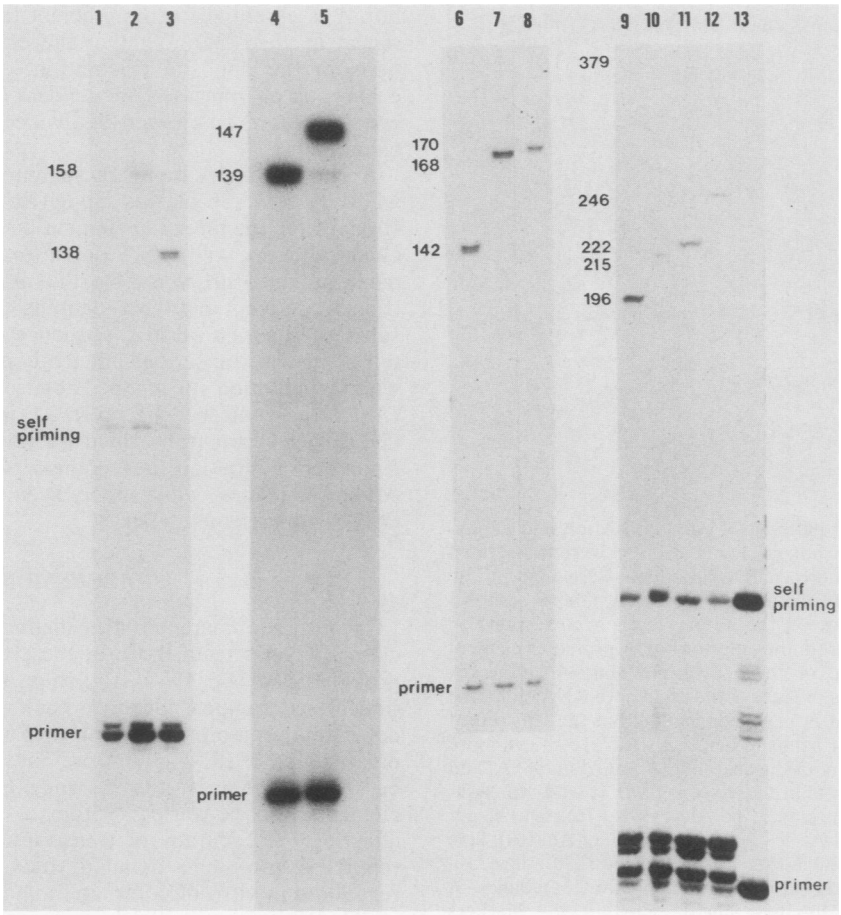

FIG. 6. Primer extension analysis of the in vitro transcripts from contraction and expansion mutants of the *Alu* repeat. The A_1 series of RNA transcripts (Fig. 5) were eluted from the denaturing gel, and their 5′ ends were mapped by the primer extension technique with the ^{32}P-end-labeled probes listed in Fig. 4. The sizes of the DNA extension products were analyzed by coelectrophoresis with DNA sequencing markers on denaturing gels and autoradiography on X-ray films. The sizes (in nucleotides) of the extension products are indicated to the left of each set of gels. Self-priming of the single-stranded 73-nt *Taq*I-*Hin*fI and 82-nt *Sau*3A-*Bst*NI probe occurred during the reactions of lanes 1 to 3 and 9 to 13, respectively. The DNA sequencing markers were derived from pBR322 and are not shown here. The transcripts mapped were derived from the following: no RNA, probe (*Taq*I-*Hin*fI fragment) only (lane 1); pd-Alu-(+37/+71) (lane 2); pd-Alu-(+63/+76) (lane 3); pd-Alu-(+63/+74) (lane 4); pd-Alu-(+56/+68) (lane 5); pi-Alu-(63) (lane 6); pi-Alu-(88) (lane 7); pi-Alu-(90) (lane 8); pi-Alu-(115) (lane 9); pi-Alu-(134) (lane 10); pi-Alu-(141) (lane 11); pi-Alu-(165) (lane 12); pi-Alu-(298) (lane 13).

1 kilobase have also been constructed by the shotgun cloning method described above and analyzed by transcription in the HeLa S-100 extract. No initiation upstream from the box A sequence could be detected in these clones by either gel electrophoresis or primer extension (unpublished data). Thus, the upper limit of the interbox spacing that allows the transcriptional initiation immediately upstream from the box A to be detected by our assays falls within the range of 300 to 600 bp. This range is similar to that of the tRNA genes (8, 18, 38).

The lower limit of this spacing can also be estimated. Because the transcriptional initiation upstream from box A occurs in clone pd-Alu-(+63/+76) but not in pd-Alu-(+37/+71) (Fig. 4 to 7), the lower limit falls between 26 and 47 bp. Furthermore, initiation upstream from the second box A-like sequence (box A′) within the *Alu* repeat is enhanced in clones pi-Alu-(88), pi-Alu-(90) and pi-Alu-(96) (Fig. 3 to 6). Because the distance between box B and box A′ of clone pi-Alu-(88) is 44 bp (Fig. 7), it can be concluded that the lower limit of the interbox spacing that allows initiation to occur upstream from the box A sequence is between 26 and 44 bp. This is somewhat different from the $tRNA^{Pro}$ gene from *C. elegans*, in which the initiation still ocurs upstream from box A, even when the two promoter boxes are separated by only 17 bp (8).

Thus, although the posterior promoter element of the *Alu* repeat by itself is capable of directing the transcriptional initiation at approximately 70 bp upstream, it cannot do so when there is a box A present within 47 to 298 bp upstream from box B (Fig. 4 and 7). Within this range of the spacing between boxes A and B, box A plays the major role in the positioning of the initiation site(s). This positioning function of box A does require cooperative interaction with box B because box A alone cannot initiate transcription (35). However, if box A is absent in *cis* (35) or is separated from box B by less than 26 bp, as in clone pd-Alu-(+37/+71) (Fig. 4 and 7), or longer than 600 bp (data not shown), the directing function of the box B-containing element becomes dominant.

Evolutionary conservation of the interpromoter element

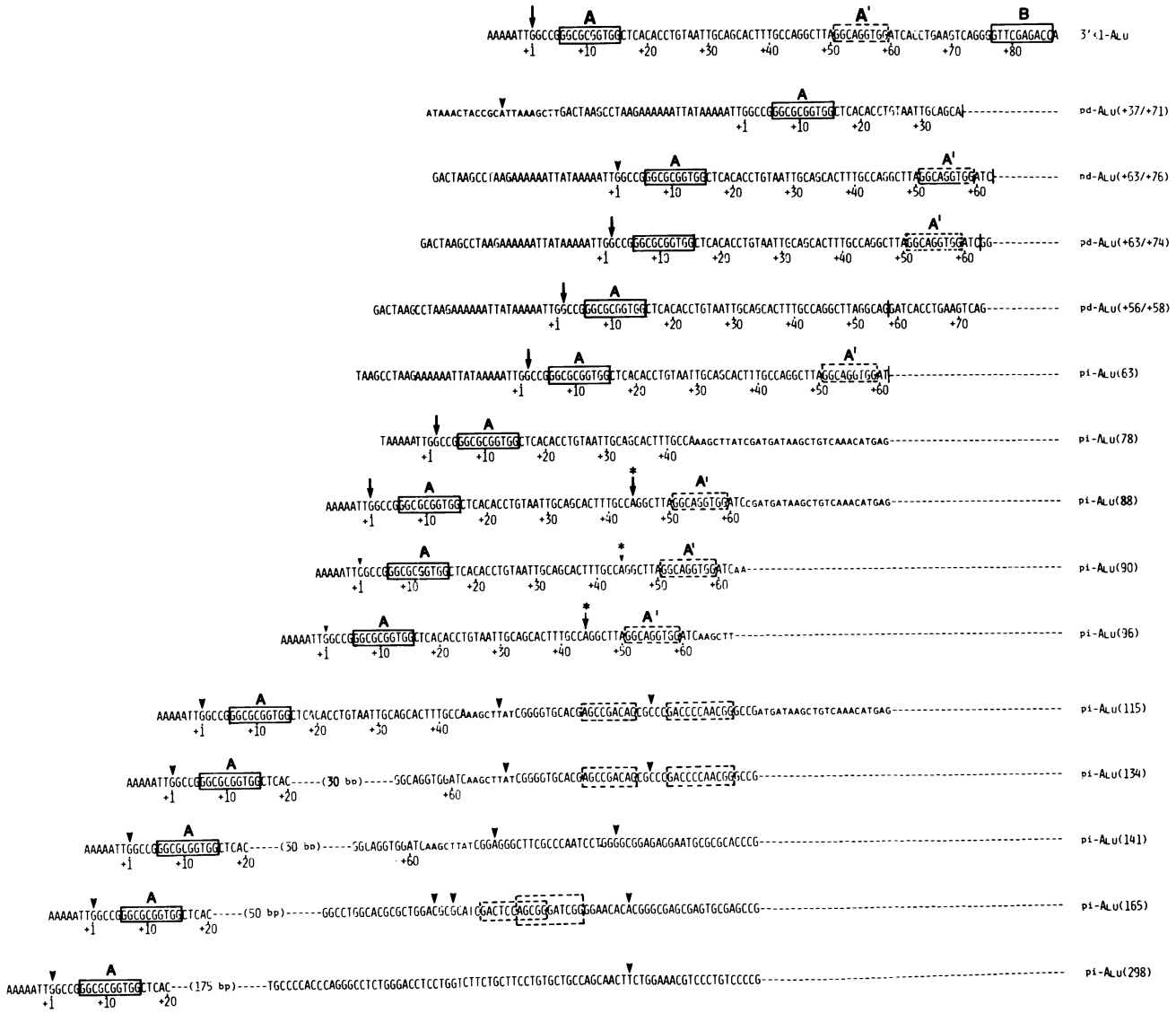

FIG. 7. Transcriptional initiation sites of the contraction and expansion mutants on the nucleotide sequence level. The DNA sequences of the contraction and expansion clones are shown below the wild-type *Alu* repeat, with their box B sequences aligned. The box A and B promoter sequences are indicated by solid boxes. Dashed boxes denote sequences homologous to box A, including box A′ which is located within the *Alu* repeat (see text). Human DNA sequences are represented by capital letters, and those of pBR322 are represented by small letters. Dashed lines represent bases identical to the sequence directly above. Numbers below the sequences are relative to the first base (base 1) of the wild-type *Alu* repeat. Deletion junctions in clones pd-Alu-(+37/+71), pd-Alu-(+63/+76), pd-Alu-(+63/+74), pd-Alu-(+56/+58), and pi-Alu-(63) are indicated by vertical lines. Transcription initiation sites mapped by primer extension analysis, as exemplified in Fig. 6, are indicated by vertical arrows.

spacing for optimum transcriptional efficiency. The changes in the efficiency of transcriptional initiation upstream from the posterior box A sequence of the *Alu* repeat, as a function of the spacing between boxes A and B, are shown in Fig. 9. The clone pd-Alu-(+63/+76) is not included in Fig. 9 because the mutations in the 5′ portion of its directing element (see sequences in Fig. 7) may have caused the low efficiency of transcription. This clone failed to compete for transcription factor(s) in a competition experiment against the wild-type gene, whereas pd-Alu-(+37/+71) was able to compete effectively, despite the fact that it was also weakly transcribed (unpublished data). It can be seen from the bell-shaped curve in Fig. 9 that the two most efficiently transcribed repeat sequences are the wild type and the mutant with an interbox spacing of 60 bp. The efficiency drops when the distance between the two promoter elements is either increased or decreased. This is consistent with the observation that transcriptional initiation upstream from box A′ is enhanced when this sequence is brought to within 44 to 52 bp upstream of box B. The low efficiency of transcription of the *Alu* mutants with shortened or lengthened spacing between the promoter elements may be the result of the inability to form stable preinitiation complexes, as has been suggested for the tRNA genes (9).

Previous analyses of the *C. elegans* $tRNA^{Pro}$ gene (8) and *Drosophila* $tRNA^{Arg}$ gene (38) have also revealed a bell-

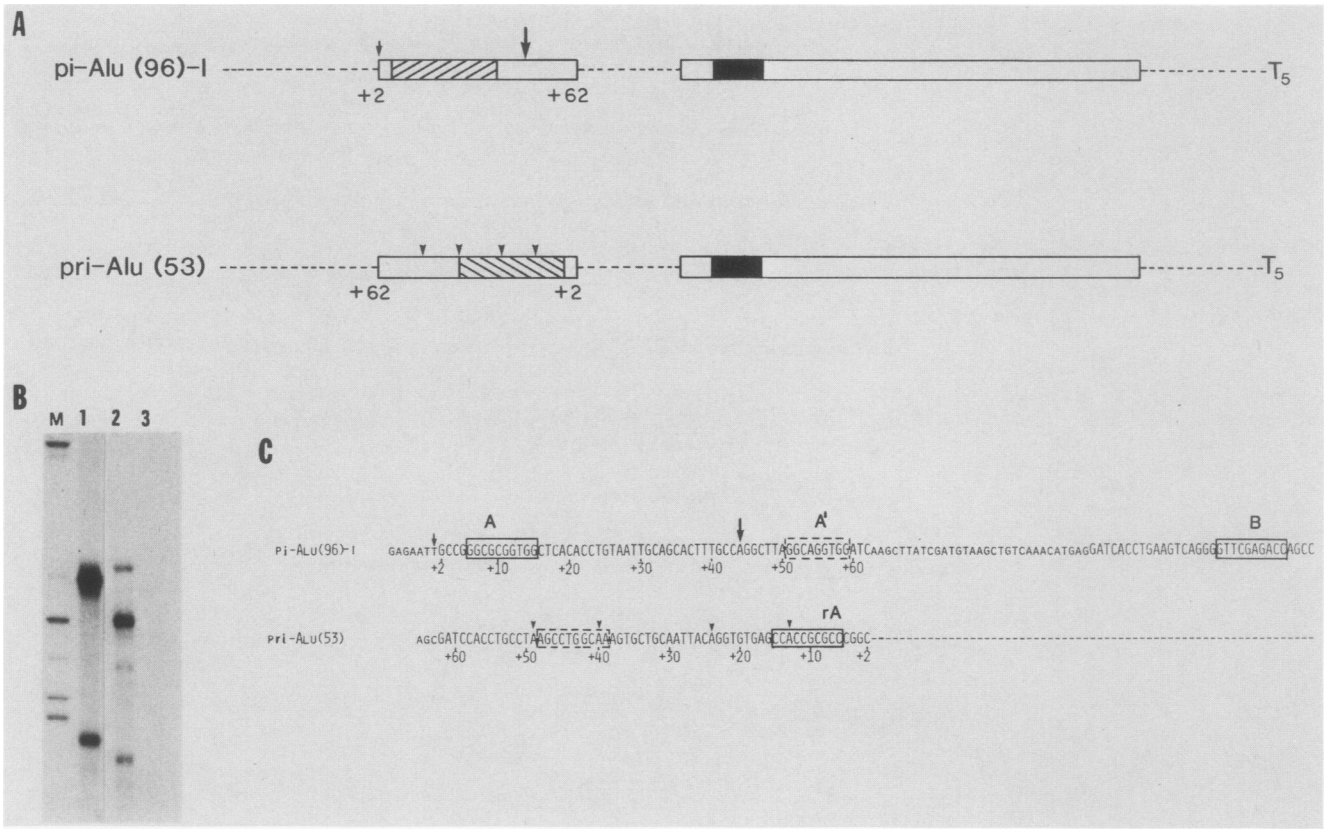

FIG. 8. Effect of reversing the orientation of enhancing element on transcription in vitro. (A) Transcription maps and promoter organization of two *Alu* repeat mutants. The clones pi-Alu-(96)-I and pri-Alu-(53) were constructed as described in the text. The open bars represent the *Alu* repeat sequence, and the dashed lines are the pBR322 sequence. The enhancing and directing elements are represented by hatched and black boxes, respectively. Note that the orientation of the enhancing element in clone pri-Alu-(53) is opposite that of the wild-type *Alu* repeat. Transcriptional initiation sites, mapped by primer extension (data not shown), are indicated by vertical arrows. Termination of transcription occurs at the cluster of five thymidines in the pBR322 vector. (B) Transcriptional patterns of clones pRBα1 (lane 1), pi-Alu-(96)-I, (lane 2), and pri-Alu-(53) (lane 3) on denaturing gels. Markers (M) used were ^{32}P-labeled, single-stranded *Hae*III-pBR322 fragments. (C) Summary of transcriptional initiation sites of clone pi-Alu-(96)-I and pri-Alu-(53) on the nucleotide sequence level. The symbols are similar to those described in the legend to Fig. 7. Box A and B scquences are indicated by solid boxes. Box A′ and other box A-like sequences are indicated by dashed boxes. The reversed box A is denoted as rA in clone pri-Alu-(53).

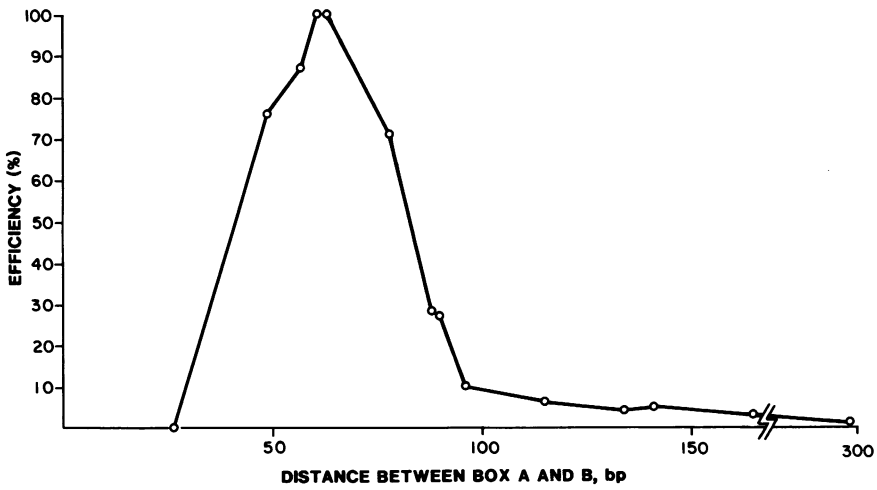

FIG. 9. Efficiency of transcriptional initiation upstream from box A as a function of the spacing between boxes A and B of the *Alu* family repeat gene. Gel pieces containing the A_1 and A_2 series of RNA bands (Fig. 5) of inidividual mutant clones were cut out, and their radioactivities were determined by the Cerenkov method. The percentages of these RNAs, relative to the amount of transcripts from the wild-type *Alu* repeat, were then estimated and plotted against the spacing between boxes A and B.

shaped dependence of the transcriptional efficiency on the interpromoter element spacing. However, the most efficiently transcribed mutants of the tRNA genes are those with the spacings between boxes A and B close to the wild-type tRNA gene, i.e., 30 to 40 bp. Thus, the *Alu* repeat and the tRNA genes have acquired certain intrinsic difference(s) in their promoter function during evolution, despite the apparent homology between their box A and B elements. This difference(s) could affect the formation, conformation, or both of the transcription complexes in such a way that each gene family is transcribed most efficiently at their current spacing of the internal promoter elements. Whether this differential pattern has evolved under selection is not known.

It should be mentioned here that in addition to the intragenic promoter elements, the transcriptional efficiency of several polymerase III-dependent genes can be modulated by upstream DNA elements (see references 6 and 46 and references therein). In particular, 7S RNA gene transcription is greatly reduced when its upstream sequence (−2 to −66) is damaged by cloning (46). This transcriptional enhancement of the 7S RNA gene, the proposed ancestral form of the human *Alu* family repeats, may account for the in vivo abundance of its transcripts when compared with the *Alu* family repeats (46). Interestingly, even though the *Alu* repeats do not have this 7S gene-specific controlling element upstream, their transcription in vitro does require the presence of a contiguous DNA helix extending into the region of −30 to −85 (35).

Factor requirement of the *Alu* repeat transcription. Results of in vitro binding and transcription studies of class III genes (5, 17, 28, 36, 39, 43, 44) have indicated that one factor, factor C (28), is essential to the formation of stable preinitiation complexes of the VAI genes. At least one other factor, factor B (28), is required for the complex formation of tRNA genes. The factor requirement of the *Alu* family repeat was not known, although the binding of factor C to its posterior promoter elements has been implicated from the sequence homology to the tRNA and VAI genes (see references 7 and 35 and references therein). Results of the transcriptional competiton experiment described in this study (Fig. 2) strongly suggest that the posterior, box B-containing promoter element of the *Alu* repeat is able to bind factor C efficiently. Furthermore, the presence of the anterior, box A-containing element enhances the ability of the *Alu* repeat to compete with the VAI gene for the transcriptional factor(s).

Experimental comparison of the *Alu* family with the tRNA and VAI genes in vitro suggests that they share many common characteristics with respect to the mechanism(s) of transcriptional initiation. These characteristics include the split intragenic promoter, factor C binding to the posterior promoter element, the cooperative interaction of the two promoter elements, the flexibility of the promoter organization, and the positioning of initiation sites by the anterior promoter element. However, the *Alu* family repeat also distinguishes itself from the other class III genes in two ways. First, the posterior promoter element, either by itself or when separated from the anterior element by short or long distances, is able to direct a relatively accurate, although inefficient, initiation. Second, the *Alu* family and the tRNA genes have evolved in such a way that each has acquired its current spacing between the two promoter boxes (approximately 60 bp for the *Alu* family and 30 to 35 bp for tRNA genes) that is optimum for transcription in vitro and possibly in vivo too. Some subtle sequence differences of the DNA templates and the resulting differential interaction with transcription factors, polymerase III, or both may be the cause for these distinctions.

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