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The Alu family repeat promoter has a tRNA-like bipartite structure

Giovanni Paolella, Miguel A.Lucero, Martin H.Murphy and Francisco E.Baralle*

Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, UK

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The essential sequences needed for Alu repeat transcription by RNA polymerase III were mapped. Experimental evidence is presented showing that the Alu repeat promoters are organised in a bipartite structure similar to the split tRNA promoters as suggested by DNA sequence homology. Furthermore, by combining fragments from efficiently and inefficiently transcribed natural Alu repeats in several recombinant clones, it was possible to investigate the regions responsible for their differences. It is clear that, apart from the short stretches of homology with the tRNA consensus sequence, there is very little constraint in the promoter sequences. However, our studies indicate that some influence on the efficiency of transcription may be exerted by regions outside the accepted promoter components.

Key words: Alu repeats/DNA sequencing/globin gene flanking region/RNA polymerase III/split promoter

Introduction

The Alu repeat sequences represent 3–6% of the human genome (Jelinek and Schmid, 1982). Their role '*in vivo*' has yet to be elucidated though a function in DNA replication and RNA processing has been suggested (Calabretta *et al.*, 1981; Jelinek *et al.*, 1980). There is also good evidence of their role in promoting gene rearrangements (Calabretta *et al.*, 1982) perhaps by generating mobile genetic elements (Calabretta *et al.*, 1982; Jagadeeswaran *et al.*, 1981) and in secretion as part of 7S RNA (Walter and Blobel, 1982; Ullu *et al.*, 1982). Alu repeats are transcribed by RNA polymerase III producing discretely sized RNAs (Duncan *et al.*, 1981; Fritsch *et al.*, 1981; Di Segni *et al.*, 1981). *In vitro*, not all the natural Alu repeats are equally efficient as templates. The different transcriptional efficiency of the two Alu repeats found in the 5'-flanking region of the ϵ-globin gene (Di Segni *et al.*, 1981; Baralle *et al.*, 1980a) provide an opportunity to study in detail the properties of the Alu repeat promoters.

The genes transcribed by RNA polymerase III contain their promoter within the coding sequence. In 5S RNA, a 30-bp region seems to contain all the information necessary for correct initiation of transcription (Sakonju *et al.*, 1980; Bogenhagen *et al.*, 1980). In the VAI gene of adenovirus an internal control region ~60 bp long could be identified (Fowlkes and Shenk, 1980; Guilfoyle and Weinmann, 1981). The transcription of tRNA genes was shown to depend on the presence of two consensus sequences of ~10 nucleotides (blocks A and B), each located ~30 nucleotides apart within the coding region (Hofstetter *et al.*, 1981; Ciliberto *et al.*, 1982a; Galli *et al.*, 1981). The Alu repeat promoters show sequences closely homologous to the conserved blocks of tRNA promoters although they are, on average, 60 nucleotides apart. However, to date, there was no experimental evidence that the Alu promoter is split. On the contrary, there was evidence suggesting that all that was necessary for transcription was a continuous region of 27 nucleotides found ~70 nucleotides from the 5' end of the Alu repeat (Fuhrman *et al.*, 1981). In this paper, we present experimental evidence that the Alu repeat promoters are organised in a bipartite structure similar to the intragenic split tRNA promoters (Hofstetter *et al.*, 1981; Ciliberto *et al.*, 1982a). Furthermore, by combining fragments from the efficiently and inefficiently transcribed repeats in several recombinant clones, it was possible to investigate the regions that may be responsible for their differences.

*To whom reprint requests should be sent

Results

Alu repeats are transcribed in vitro with different efficiency

We have previously shown that the two Alu repeats present in the 5'-flanking region of human ϵ-globin gene (see Figure 1) are selectively transcribed '*in vitro*' by RNA polymerase III (Di Segni, 1981). The repeat located farthest from the ϵ-globin gene (repeat II, Figure 1) was 2–3 orders of magnitude more efficient as an RNA polymerase III template than its companion (repeat I). This can be easily appreciated from lanes I and II, Figure 1A. Lane I is the fractionation of the RNA polymerase III transcripts produced by the pϵ 1.8 subclone containing repeat I (Di Segni *et al.*, 1981; Baralle *et al.*, 1980a, 1980b). Lane II is the result obtained using pϵ 3.7, a subclone containing repeat II. The discrete RNA molecules were produced by termination in naturally occurring terminators in the unique sequence region downstream of the repeats (see sequence data in Di Segni *et al.*, 1981). Hybridisation experiments showed that the total transcripts observed in lanes I and II were originated in the Alu repeat and did not contain a significant component of vector sequences. In Figure 2, lines 1 and 2 indicate the relevant sequences of repeats I and II. The origin of transcription has been mapped by S1 experiments (not shown) to a region around positon 0, in agreement with data previously published by Duncan *et al.*, 1981.

Characterisation of the essential sequences of the repeat II promoter

The *Bgl*II fragment a and the partial *Hae*III fragment b (see restriction map in Figure 1, and relevant sequences in Figure 2, lines 1 and 2) were subcloned in M13mp9 (Messing and Vieira, 1982) and M13mp701 (Bentley, 1982), an analogue of M13mp8 (see Materials and methods for details of the subclone construction). These subclones, when tested as templates for RNA polymerase III, produced the RNA transcripts shown in Figure 1B lanes a and b. It can be seen that fragment a and even the partial *Hae*III fragment b, only 91 bp long (see Figure 2), promotes efficient transcription. The termination of transcription in these subclones occurs in poly(T) tracks present in the M13 sequence on either side of the cloning sites (Messing *et al.*, 1981; Van Wezenbeek *et al.*, 1980). Some of these poly(T) tracks are very efficient ter-

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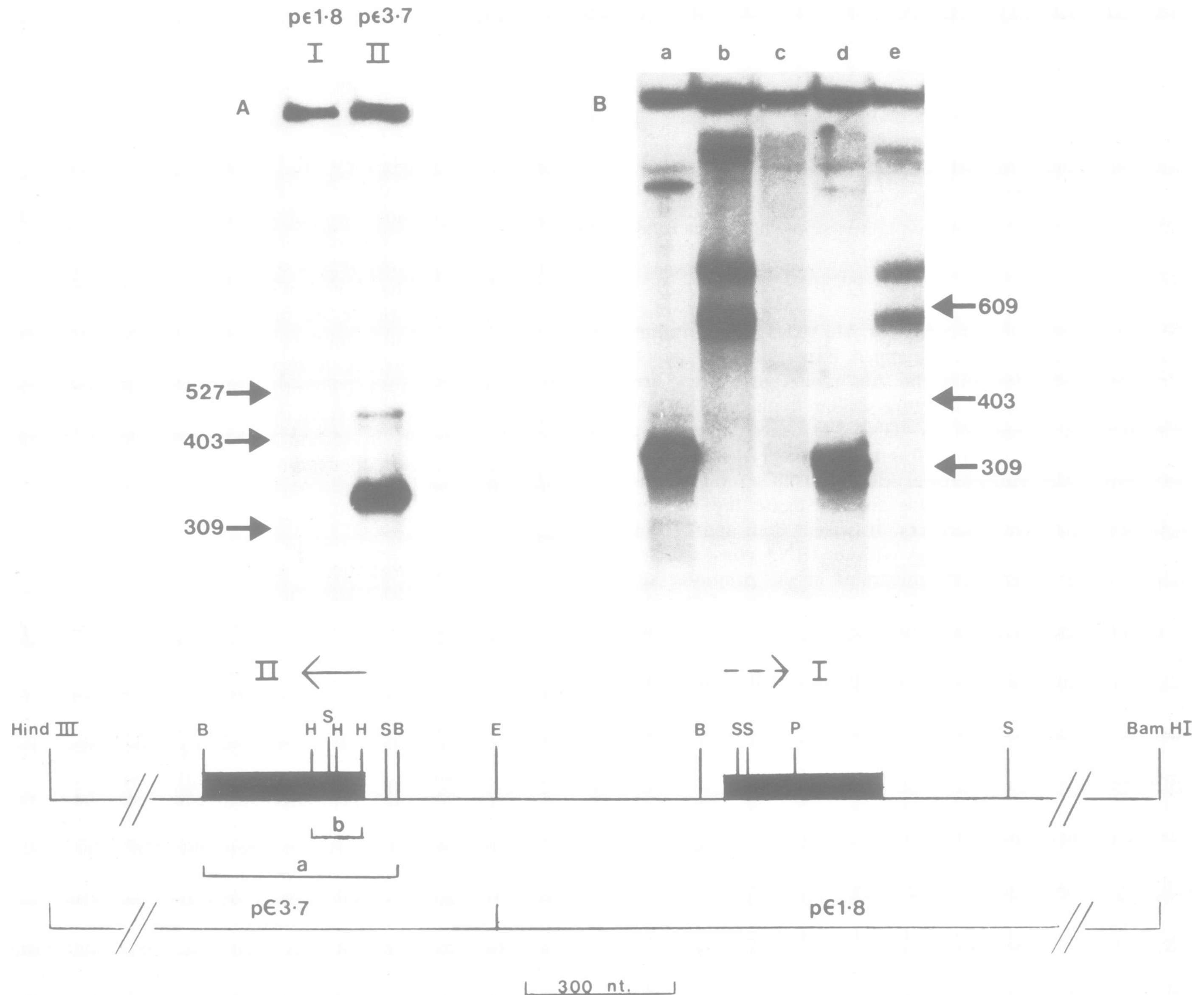

Fig. 1. Characterisation of the Alu repeat promoters found in the 5′-flanking region of ε-globin genes. The 5′-flanking region of ε-globin gene is schematically represented at the bottom of the Figure. The full boxes represent the Alu repeats I and II, the arrows indicate the direction of RNA polymerase III transcription '*in vitro*'. Relevant restriction enzyme sites are indicated as follows: B, *Bgl*II; H, *Hae*III; S, *Sau*3A; E, *Eco*RI; P, *Pvu*II. The subclones pε 3.7, pε 1.8 and the complete nucleotide sequence of the region have been previously described (Di Segni *et al.*, 1981; Baralle *et al.*, 1980a, 1980b). a and b respectively denote the *Bgl*II 382-bp fragment and the partial *Hae*III 91-bp fragment. **(A)** Autoradiograph of a 6% acrylamide 7 M urea gel fractionation of RNA polymerase III transcripts from repeats I and II. **(B)** Characterisation of the essential sequences of the repeat II promoter. The *Bgl*II fragment a and the partial *Hae*III fragment b (see restriction map above and relevant sequences in Figure 2, lines 1 and 2) were subcloned. Clones a and b contain, respectively, fragments a and b (see Materials and methods and Figure 2). The templates used were: **(a)** clone a, **(b)** clone b, **(c)** *Eco*RI restricted clone b, **(d)** *Eco*RI restricted clone b mixed with clone a, **(e)** *Eco*RI restricted clone b and then religated.

minators while other T-rich segments are 'leaky' terminators. This may explain the presence of more than one transcript from the same initiation site (Figure 1B, lanes b and c; see also Di Segni *et al.*, 1981). The promoter function of fragment b persists also when it is cloned in the opposite orientation with respect to the M13 sequences (data not shown). It seems then unlikely that the M13 sequences could contribute to the promoter function.

The integrity of the DNA template near the transcription initiation site is, however, essential for promotion. The clone carrying fragment b contains an *Eco*RI site (from the mp9 vector), three nucleotides 5′ of the CC sequence of the position 7 *Hae*III site (line 2, Figure 2). Restriction of this clone with *Eco*RI yields a linear DNA molecule, one end of which coincides with the transcription initiation site. When this linear DNA is used as template for RNA polymerase III, no transcription is observed (lane c, Figure 1B). This effect is not due to inhibitors associated with the cleaved DNA because, when equal amounts of fragment a and linear fragment b subclones are mixed, transcription of the latter occurs normally, as can be seen in lane d. The lack of transcription is not related to the fact that DNA is linear, as templates cleaved at the 3′ end of the repeat are efficiently transcribed into discrete run-off products of the expected size (see below). Furthermore, when the cleaved fragment b clone is religated, its template activity is fully restored (lane e, Figure 1B). The observed abolition of transcription when the fragment b clone is restricted with *Eco*RI is difficult to explain. One

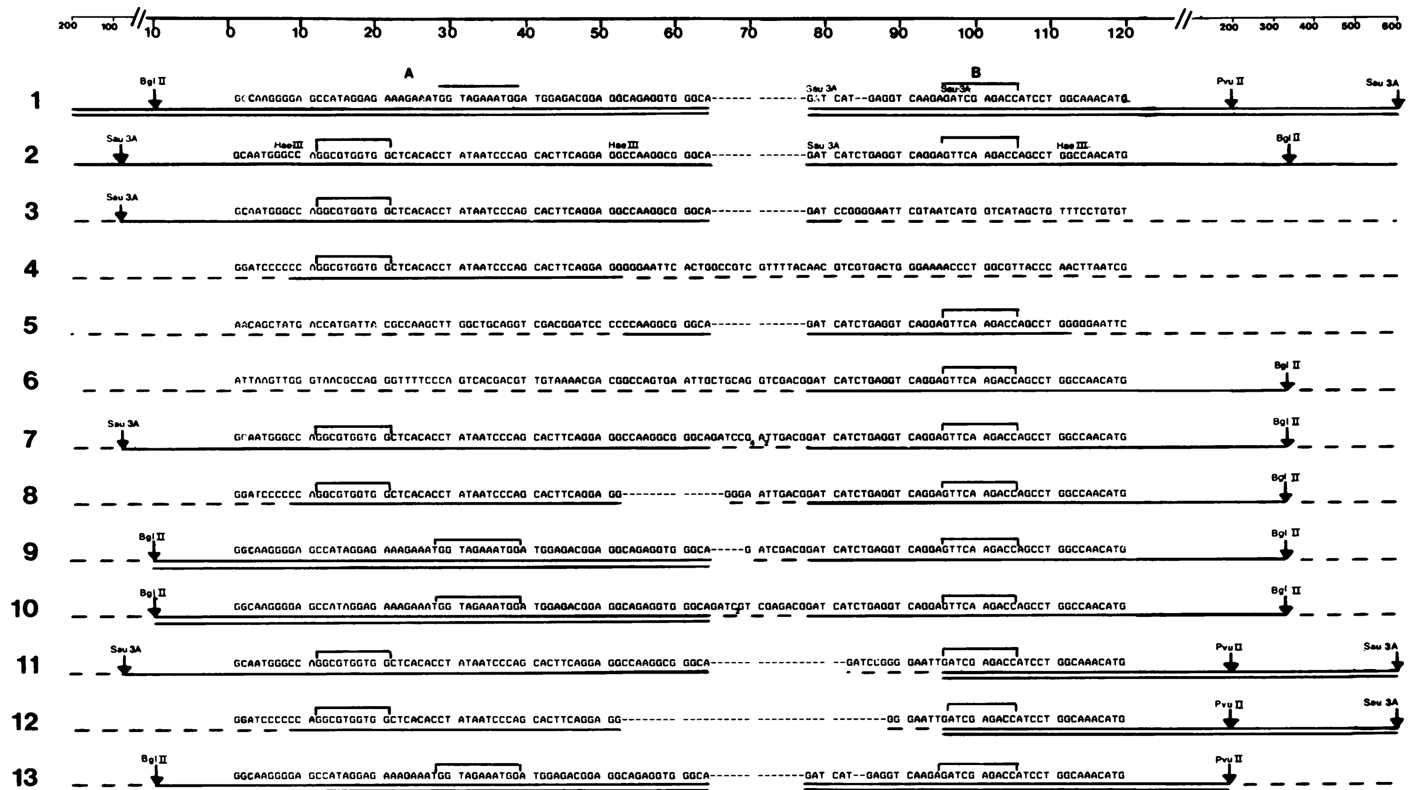

Fig. 2. Nucleotide sequence of the Alu repeat promoters and the variations constructed. The DNA sequences corresponding to the RNA transcript are shown. The molecules read 5′ to 3′ from the left of the Figure. ═ indicates repeat I sequences, —— indicates repeat II sequences and ----- indicates M13 sequences. The sequence is continuous from 0 to 120, the central gap (small dashes) was introduced to facilitate alignment. Lines 1 and 2 show the sequence of repeat I and II, respectively, as found in pε 1.8 and pε 3.7 (Di Segni *et al.*, 1981). The restriction enzyme sites used in the construction of the subclones are indicated. The subclones 3–13 were constructed inserting the relevant fragment into one of the unique restriction enzyme sites of the M13 vectors (Messing and Vieira, 1981; Bentley, 1982) (see Materials and methods). For our analysis we have divided the Alu promoter sequence in regions A and B. They are defined by the sequences to the left and right, respectively, of the *Sau*3A restriction site present at position 78 in the figure. Homologies to the consensus sequence of the tRNA promoters, namely block A: RGYNNRRYGG and block B: G$^{A}_{T}$TCRANNC are overlined in the figure. For repeat I (line 1), block A is at positions 29–39 and block B is at positions 96–105. For repeat II (line 2), block A is at position 12–21 and block B at position 96–105. Other homologies can be found in these regions, especially for block A, and only further detailed structural analysis may elucidate which is the functional one.

trivial reason may be a strong binding of the RNA polymerase to the free end of the template that coincides exactly with the initiation site. This binding may prevent the displacement of the enzyme along the template and hence the initiation of transcription.

The Alu repeat promoter is split

The two blocks of sequences that are homologous to the consensus sequences found in the tRNA promoter are located between positions 12 and 21 (block A) and 96 and 105 (block B) in line 2, Figure 2. To obtain experimental evidence for the bipartite structure of the Alu repeat promoter, a series of subclones was constructed where the block A and block B containing regions of the promoter were split at different points. In these experiments, we will refer to the two 10 nucleotide sequences homologous to the tRNA promoters as blocks A and B (boxed in Figure 2) and we shall use the terms region A and B for the sequences left and right respectively of the *Sau*3A restriction site present at positon 78 (see Figure 2 lines 1 and 2). Clone 3 contains a 110 bp long *Sau*3A fragment from repeat II including the block A; clone 4 is a shorter version of this segment consisting of a 44 bp long *Hae*III fragment. Clones 5 and 6 are respectively a 47-bp *Hae*III fragment and 248-bp *Sau*3A/*Bgl*II fragment, both including the block B consensus sequence. These four subclones were not significantly transcribed by RNA polymerase III as can be seen in Figure 3A, lanes 4, 5, 3 and 6. The Alu promoter was reconstructed by cloning the relevant fragments in the *Hinc*II site of clone 6, which contains region B. Fragments containing region A were obtained from clones 3 and 4 (see Materials and methods), thus restoring the block A and B to a single molecule (clones 7 and 8, Figure 2) in a slightly altered relationship. In the original repeat II, the first nucleotide of blocks A and B are 71 nucleotides apart, in clones 7 and 8, they are 88 and 70 nucleotides apart, respectively, the spacer nucleotides being the sequence originally present in M13 between the cloning sites. Clone 8 also has a deletion of the sequences originally present in the Alu repeat between positions 52 and 64 (Figure 2). Figure 3B, lanes 7a and 8a, shows the products obtained by RNA polymerase III transcription of clones 7 and 8. It is clear that the promoter activity has been fully restored. The main RNA band is of the size expected (~580 nucleotides) if termination occurs in the poly(T) tract present in the M13 flanking region. The weaker RNA bands between the size markers 344 and 506 may represent premature termination in some of the T-rich sequences present in the M13 genome before the poly(T) track. This assumption is supported by the fact that all of them moved to a single run-off product when cleaved with *Eco*RI (see below). A similar phenomenon was observed using the intact Alu repeat template (Di Segni *et al.*, 1981, Figure 3A). Clones 7 and 8 were then cleaved with the restriction enzyme *Eco*RI.

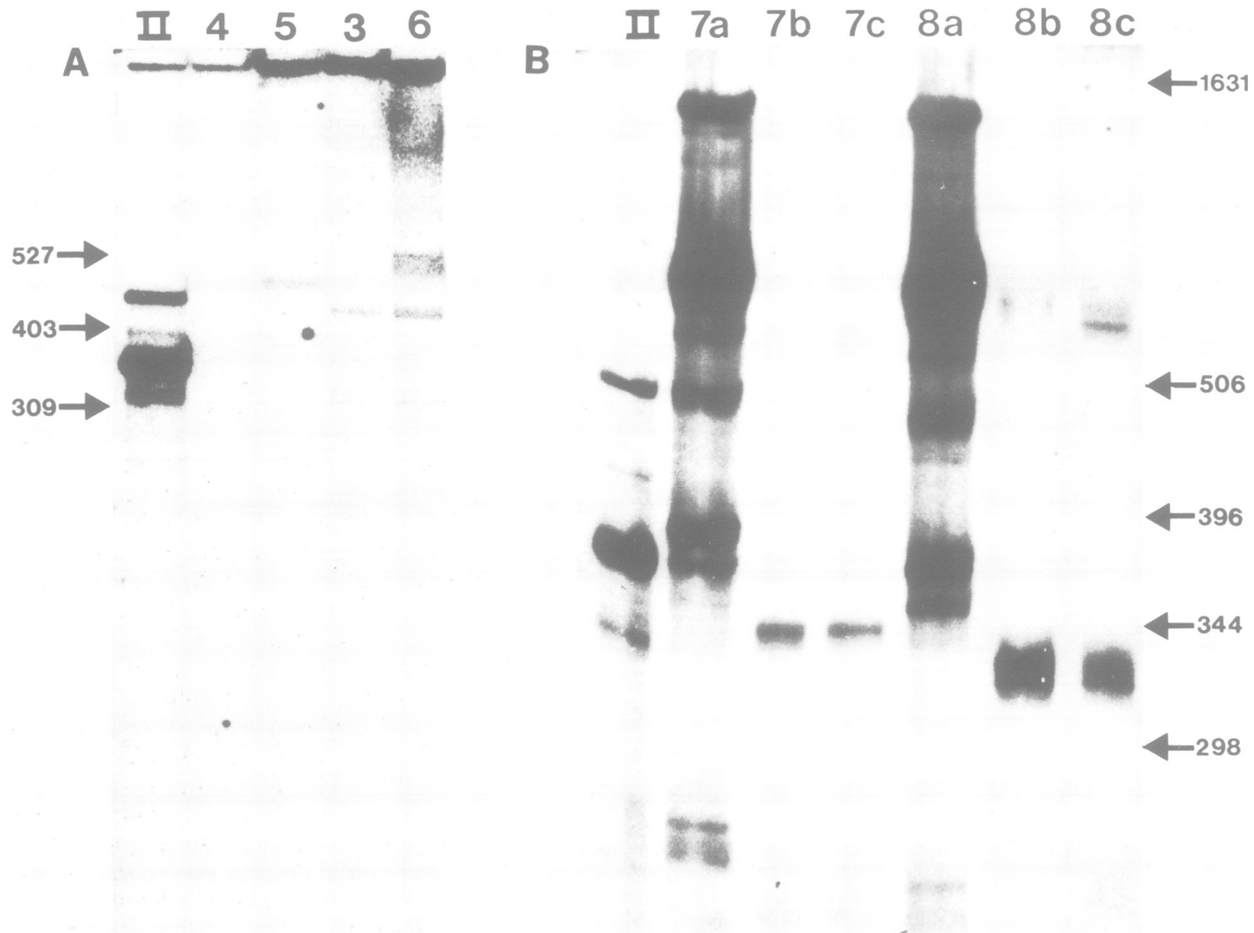

Fig. 3. Demonstration of the bipartite structure of the Alu repeat II promoter. **(A)** *In vitro* transcription of subclones containing either only region A or only region B. **Lane II** is a positive control using as template pε 3.7 (intact repeat II). **Lanes 4, 5, 3** and **6** show the transcription efficiency of clones 4, 5, 3 and 6 (Figure 2). DNA concentrations and inhibitory effects of the templates were ruled out by carrying out DNA concentration curves and template mixing experiments (not shown). **(B)** *In vitro* transcription of subclones constructed from those analysed in **(A)** as described in Materials and methods and Figure 2. **Lane II** shows pε 3.7 transcripts; **lanes 7a** and **8a** show the RNAs obtained from clones 7 and 8 (Figure 2). **Lanes 7b, 7c** and **8b, 8c** show the transcripts obtained when the templates used were clones 7 and 8 cleaved with *Eco*RI. The RNAs produced were confirmed by hybridisation experiments to have originated from the Alu repeats, the transcription initiation site being around position 0 as deduced from the run-off products **(lanes 7bc** and **8bc)** (Figure 2). Single strand DNA size markers are indicated.

This produced a linear molecule. The RNA polymerase III transcript will terminate by running off the template at the cleaved restriction site and the predicted RNA size will be 340 nucleotides for clone 7 and 322 nucleotides for clone 8, assuming that the position of the transcription initation site is determined by region A, as seems to be the case in the tRNA genes (Hofstetter *et al.*, 1981; Hall *et al.*, 1982; Ciliberto *et al.*, 1982b). Figure 3B, lanes 7bc and 8bc, shows the transcription products of clones 7 and 8 cleaved with *Eco*RI. The main RNA bands are entirely consistent with the size predicted. The 5′ end of the RNA molecules was not defined precisely and initation may occur at one or more places ± 10 nucleotides from positon 0 (Figure 2). This may be the reason for the observation of two or more contiguous bands in the run-off products. It is clear then that sequences inserted 3′ of the A block will increase the length of the transcript, but will not alter the initiation site. It should be noted that in clone 8, the nucleotides between position 52 and 64 of the original repeat II are deleted (see lines 2 and 8, Figure 2). The 12 nucleotides are well conserved in the Alu repeats, but they are certainly not essential for transcription as the efficiency of clone 8 transcription is of the same order of magnitude as the original repeat II.

Repeat I and II promoters can interchange region A

There is no significant homology between region A of repeat I and II, and even the block A segment differs in nucleotide sequence and position (see Figure 2 and Di Segni *et al.*, 1981). It was proposed that this lack of homology may be the cause of the low efficiency of transcription in repeat I (Di Segni *et al.*, 1981). To test this hypothesis, hybrid repeats were constructed. Figure 2, lines 9, 10, 11 and 12, show different combinations of repeats I and II restriction fragments joined together (see Materials and methods). Clones 9 and 10 have repeat I region A separated from repeat II region B by 20 and 26 nucleotides, an essentially similar configuration to clones 7 and 8 discussed above. In clones 11 and 12, containing repeat II region A and repeat I region B, we were unable to preserve the region B sequences between position 77 and 95 (Figure 2) with the construction strategy used (*Sau*3A cleavage). These sequences were partly replaced by M13 sequences. Region A and region B were thus closer to each

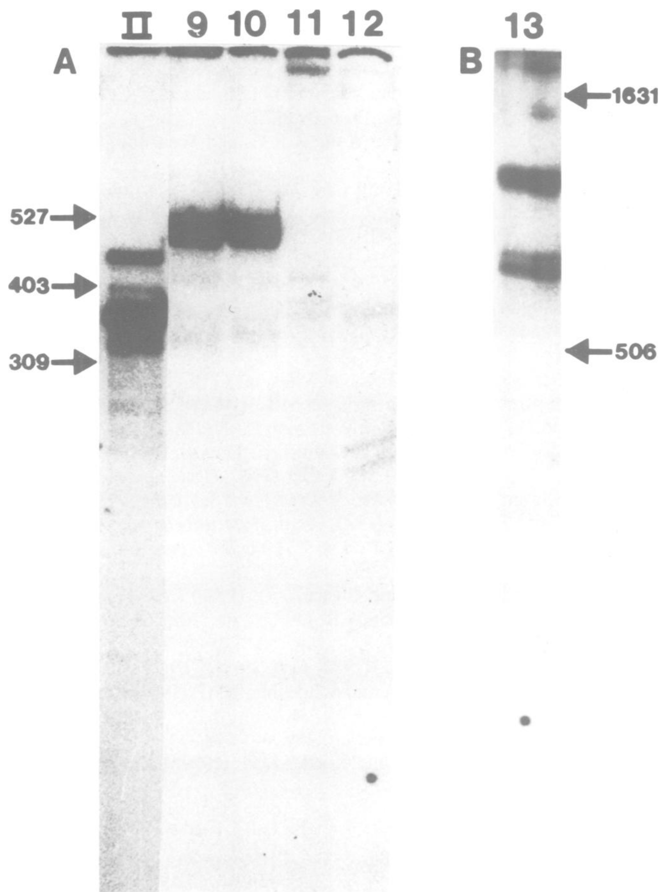

Fig. 4. Transcription of repeat I-repeat II hybrid promoters. **(A)** *In vitro* transcription of: **lane II** pε 3.7, **lanes 9** and **10**, clones 9 and 10 (see Figure 2) containing region A from repeat I and region B repeat II. **Lanes 11** and **12** clones 11 and 12 (Figure 2) containing region A from repeat II and region B from repeat I and **lane 13** clone 13 (Figure 2) a reduced version of repeat I. The origin of the transcripts and their initiation site were confirmed as described in Materials and methods.

other than in the original repeats but still at a distance known to be functional in tRNA promoters (Hofstetter *et al.*, 1981; Ciliberto *et al.*, 1982a).

Clones 9 and 10 carrying region A from the inactive repeat I, and region B from the active repeat II, were shown to be transcriptionally active at levels comparable with intact repeat II (see Figure 4A, lanes 9 and 10). On the other hand, clones 11 and 12 carrying block B from the inactive repeat I and block A from the active repeat II were inactive as templates. This was a surprising result because all the structural homologies indicated a functional promoter (see Discussion). The main difference between clones 11 and 12 and clones 9 and 10 was that the former pair contained extensive extra sequences including the 3′ region of the Alu repeat and its flanking region. We tested the effect of these sequences by modifying repeat I. Clone 13 (Figure 2) is a shorter version of repeat I lacking all the 3′ half of the Alu repeat. Its transcription produces discrete size RNAs (see Figure 4, lane 13). This is not simply an effect of better termination as only small quantities of RNA were produced by repeat I, as tested by direct hybridisation of the total transcription products against Alu repeat sequences. This is also evident in the autoradiograph shown in Figure 1, lane 1. The most likely explanation is that the sequences 3′ of the *Pvu*II site (Figure 2) are inhibitory. A similar effect was observed with 5′-flanking sequences of some tRNA genes (De Franco *et al.*, 1981). Further detailed work is needed to establish the nature of the sequences involved and the reason for the inhibition.

Discussion

The experiments described above definitely showed that the Alu repeat promoters have a bipartite structure similar to the tRNA gene promoters. The regions necessary for repeat II transcription are defined as follows: region A between nucleotides 9 and 52, and region B between nucleotides 78 and 112. The distance between the two regions, 12 nucleotides in repeat II (line 2, Figure 2), 29 nucleotides in clone 7 and 11 nucleotides in clone 8, and the nucleotide sequence of the spacer are not critical within the limits tested (see Figure 2). Region A appears to be responsible for the position of the RNA polymerase III at the transcription initiation site. If sequences are inserted 3′ of the block A (as in clones 9 and 10, Figures 2 and 3), the length of the transcript increases, but the 5′ end of the molecules maps around the same initiation site. This is similar to the tRNA promoters where the initiation site is a purine 10–20 nucleotides upstream from the first nucleotide of block A (Hall *et al.*, 1982). Our data also suggest that this is not the only function of region A because the efficiency of total transcription (regardless of where it is initiated) is greatly decreased if region A is not present (see Figure 3). Fuhrman *et al.* (1981) analysed the RNAs generated by an RNA polymerase transcription of a collection of clones containing Alu repeat sequences. Their data seem to indicate that region A was not essential for transcription and only nucleotides 83–110 (in our numbering, Figure 2) were needed. This discrepancy may be the result of either region A-like sequences present in the 5′-flanking regions of their inserts or of different properties of the enzyme preparations.

Of course, the most likely essential feature within regions A and B are the blocks A and B homologous to the ones present in the tRNA gene promoters. A great variability of the sequences surrounding block A does not seem to affect promoter function, as shown by the fact that region A from repeat I was fully active, although it shows scant homology with region A from repeat II.

The tRNA promoter studies have already shown that the lack of homology in region A will not matter as long as some form of block A sequence is present. Hence, the observation that clones 9 and 10 are fully active is easy to accept. It is more difficult to explain the lack of activity in clones 11 and 12. Repeat I region B has a very good block B sequence and the only difference is found in the few nucleotides 5′ of it. There is, though, an extensive sequence 3′ of the block B originated from the flanking region of ϵ-globin gene (see Figures 1 and 2, and Di Segni *et al.*, 1981; Baralle *et al.*, 1980a). The lack of homology between positions 77 and 95 is unlikely to influence transcription; similar differences to the ones present between repeat I and II are found in numerous active Alu promoters (Jelinek and Schmid, 1982) and tRNA promoters (Hofstetter *et al.*, 1981; Ciliberto *et al.*, 1982a; Galli *et al.*, 1981; Ciliberto *et al.*, 1982b). However, it cannot be ruled out that the differences found between clones 11 and 12 and repeat I immediately 5′ of block B are responsible for the abolition of transcription. On the other hand, our data suggest that the sequences 3′ of the block B in repeat I do have an inhibitory effect on transcription. The repeat I promoter was fully active after the removal of the 3′ sequences,

as shown by the transcripts produced by clone 13 (Figures 2 and 4).

It has been firmly established that the Alu repeat promoter has a bipartite structure similar to tRNA gene promoters. The two regions of the promoter present stretches of sequence 10 – 11 nucleotides long that are homologous to the consensus sequence for blocks A and B of the tRNA promoter. It has been shown that the distance between them and the sequence of the spacer can be varied (within certain limits) without affecting the promoter function. However, other sequences may influence the efficiency of transcription, as shown by the activation of the repeat I promoter by the removal of the 3′ end of the repeat I. Further experiments are needed to define more precisely the boundaries of the essential regions.

Materials and methods

Subclone construction

The primary sources of all subclones were the plasmids pϵ 1.8 and pϵ 3.7 which contain repeat I and II, respectively, and have been previously described (see Figure 1) (Baralle *et al.*, 1980a, 1980b; Di Segni *et al.*, 1981). The subclones a, b and 3 – 13 (Figures 1 and 2) were constructed using the M13 vectors mp9 and mp701 as follows: clone a, *Bgl*II fragment a into the *Bam*HI site of mp701; clone b, partial *Hae*III fragment b into the *Sma* site of mp9; clone 3, *Sau*3A fragment into the *Bam*HI site of mp701; clone 4, *Hae*III fragment into the *Sma*I site of mp9; clone 5, *Hae*III fragment into the *Sma*I site of M13mp9; clone 6, *Sau*3A/*Bgl*II fragment into the *Bam*HI site of mp701; clone 7, *Eco*RI (filled in)/*Hinc*II fragment from clone 3 cloned in the *Hinc*II site of clone 6; clone 8, *Eco*RI (filled in)/*Hinc*II fragment of clone 4 cloned into the *Hinc*II site of clone 6; clone 9, *Bgl*II/*Sau*3A (filled in) fragment of clone 1 cloned into the *Hinc*II site of clone 6; clone 10, the *Bgl*II/*Sau*3A fragment of clone 1 was cloned in the *Bam*HI site of mp701, excised from it with *Eco*RI and *Sal*I, filled in and cloned into the *Hinc*II site of clone 6; clone 11, *Sau*3A fragment from clone 2 filled in and cloned in the filled in *Eco*RI site of clone 3; clone 12, the *Sau*3A fragment of clone 1 filled in and cloned into the filled in *Eco*RI site of clone 4; clone 13, *Bgl*II/PvuII fragment of clone 1 cloned into the *Sma*I site of mp9.

All the clones were sequenced by the dideoxy technique (Sanger *et al.*, 1977) to confirm their structure.

In vitro transcription of the clones and analysis of the products

The source of RNA polymerase III used was an S100 supernatant from HeLa cells prepared according to Weil *et al.* (1979). All the DNAs tested as template were titrated up to 3 μg of DNA in a 25 μl reaction mixture containing 15 μl of S100 supernatant, 0.5 mM ATP, CTP and UTP, 0.05 mM GTP, and 5 μCi of [α-^{32}P]GTP (Amersham International, sp. act. 410 Ci/mM), 1 mM creatine phosphate and 0.1 mM EDTA. The reaction mixtures were incubated for 60 min at 30°C and then processed according to Birkenmeier *et al.* (1978). The RNA products were then fractionated by electrophoresis on a 6% acrylamide 7 M urea gel. The transcription initiation site and its direction were mapped by hybridisation to appropriate single strand M13 clones and by S1 digestion of the hybrids.

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