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Construction and functional analysis of a series of synthetic RNA polymerase III promoters

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[Plates 1 and 2]

RNA polymerase III promoters were constructed by cloning chemically synthesized double stranded analogues of the box A and box B consensus sequences into suitable vectors. In contrast to approaches adopted previously for the analysis of RNA polymerase III promoters, this method has no limitation on the structure and number of variants generated, and allows critical sequences in various permutations to be studied. Furthermore, the series of synthetic polymerase III promoters created constitute a collection of point mutation variants and hence provide a powerful tool for the analysis of nucleotides essential for promoter function. The results demonstrate that these two boxes, when separated by approximately 50 base pairs, are sufficient to direct efficient transcription, and that substitution of certain nucleotides causes reduced template activity.

Introduction

The genes transcribed by RNA polymerase III contain their promoter within the coding sequence (Telford *et al.* 1979; Sakonju *et al.* 1980; Fowlkes & Shenk 1980). The transcription of tRNA, VAI and Alu repeat genes by RNA polymerase III has been shown to be dependent on the presence of two intragenic regions which contain the consensus sequences RGYNNRRYNGG (box A) and G$^{\mathrm{A}}_{\mathrm{T}}$TCRANNC (box B) located 30–60 nucleotides apart (Galli *et al.* 1981; Hofstetter *et al.* 1981; Guilgoyle & Weinmann 1981; Paolella *et al.* 1983). However, variation has been observed in the relative importance of these and 5′ sequences in different systems (Sharp *et al.* 1981, 1983; Carrara *et al.* 1981). The boxes were first noted by comparison of sequence homologies between all functional RNA polymerase III promoters, while the boundaries of regions essential for transcription were defined by experiments with cloned genes deleted by restriction enzyme action or treatment with exonucleases. The limitation of these approaches is that deletion and insertion mutants inevitably juxtapose DNA sequences not normally next to each other, or change the spatial relationship of the remaining sequences. Additional evidence for the sequence requirements of RNA polymerase III promoters has been derived from the study of point mutations in tRNA genes which alter template activity. The isolation of these mutants has been dependent on either biological assays (Koski *et al.* 1980) or chemical mutagenesis (Ciampi *et al.* 1982; Traboni *et al.* 1982; Folk & Hofstetter 1983) and therefore were limited in the types of mutations that could be produced.

To overcome these experimental limitations, we chose to test directly the role of these consensus sequences by cloning chemically synthesized double-stranded analogues of the boxes

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into suitable vectors. In this way, the relative importance of the individual components of a functional promoter with variations in the consensus boxes could be assessed in an *in vitro* RNA polymerase III transcription system.

Materials and methods

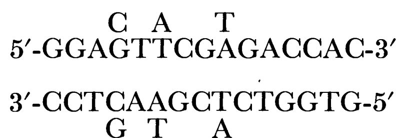
Oligonucleotide synthesis

The mixture of double stranded oligonucleotides related to box A was synthesized as the
G T A C
complementary strands 5′-TGGCATAGTTGGCT-3′ and 5′-AGCCAACTATGCCA-3′, and the mixture of double stranded oligonucleotides related to box B was synthesized as the
C A T A T G
complementary strands 5′-GGAGTTCGAGACCAG-3′ and 5′-CTGGTCTCGAACTCC-3′ by the solid phase phosphotriester method (Gait *et al.* 1980). We included the bases flanking either side of the box consensus sequences to allow for a selective hybridization screen.

Clone construction

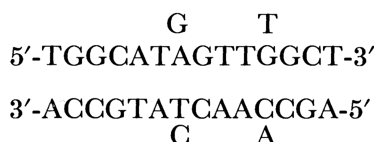
In all cases, the duplexes were unphosphorylated to avoid the formation of concatamers, and a hundred-fold molar excess of oligo-duplex over vector was employed. Ligations were performed at room temperature overnight in 10 μl reaction mixtures containing 50 ng vector, 10 ng oligo-duplex, 5 units T4 ligase, 1 mM ATP, 50 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 12 mM DTT.

The M13 recombinants 1 and 2 (see figure 1) have been described (Paolella *et al.* 1983). Clones 3–10 were constructed by cloning the chemically synthesized box B duplexes



into the filled-in *Eco*R1 site of clone 2. The correct orientation of box A with respect to box B was selected by a hybridization between the phage plaques and the complementary oligonucleotide (Wallace *et al.* 1981). The positive clones were sequenced by the dideoxy method (Sanger *et al.* 1977).

The chemically synthesized box A duplexes



and box B duplexes were cloned into the *Hin*cII or *Sma*I site of M13mp9 (Messing & Vieira 1982) respectively, to generate the precursors of the total synthetic promoter (see steps 1 and 2, figure 3).

The required recombinants of box B were selected by hybridization and characterized by dideoxy sequencing. Successful box A only recombinants were isolated by a combination of the M13 β-galactosidase screen (Messing & Vieira 1982) and dideoxy sequencing. Isolation of recombinants containing both box A and box B (figure 3, step 2) was more problematical

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```
Hae III  box A                              Hae III      Sau 3A          box B     Hae III
GGCCAGGCGTGGTGGCTCACACCTATAATCCCAGCACTTCAGGAGGCCAAGGCGGGCAGATCATCTGATCAGGAGTTCAAGACCAGCCTGGCC   1
                                                                  GG

                                                                       Eco RI
GGCCAGGCGTGGTGGCTCACACCTATAATCCCAGCACTTCAGGAGGCCAAGGCGGGCAGATCcggggaattcgtaatcatggtcatagctgtt   2

                                                                [GGAGTTCGAGACCAG]                3
                                                                [GGAGTTCGTGACCA-]                4
                                                                [GGACTTCGAGACCAG]                5
                                                                [GGAGTACGAGACCAG]                6
GGCCAGGCGTGCTGGCTCACACCTATAATCCCAGCACTTCAGGAGGCCAAGGCGGGCAGATCcggggaatt               aattcgt
                                                                [GGAGTACGTGACCAG]                7
                                                                [GGACTTCGTGACCAG]                8
                                                                [GGACTACGTGACCAG]                9
                                                                [GGAGTTCGAGACC--]                10

                                                                 G(A/T)TCRANNC          box B
                                                                 123456789
```

FIGURE 1. Nucleotide sequence of the Alu repeat promoter 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. Clone 1 is the fully active natural Alu repeat promoter; boxes A and B are indicated. Clone 2 is a derivative of clone 1 where box B is deleted; the M13 vector sequence is in lower case type. Clones 3–10 were constructed as described in section on materials and methods. The box B sequence permutations of each recombinant isolated are indicated within the brackets. Variations from the wild type sequence are in bold type. Small dashes have been introduced to facilitate alignment. The relevant restriction enzyme sites are indicated.

FIGURE 3. Overall strategy for the construction of the synthetic RNA polymerase III promoters. H, B and S are the restriction sites *Hinc*II, *Bam*HI and *Sma*I respectively in M13mp9. Step 1: the mixture of double stranded box B-related oligonucleotides was cloned into the *Hinc*II site. Step 2: the mixture of double stranded box A-related oligonucleotide was cloned into the *Sma*I site of the wild type box B recombinant. Step 3: a 39 base pair spacer was cloned into S1 nuclease treated *Bam*HI of the box A variants generated in step 2. The predicted direction of transcription is indicated (→).

as plaque hybridization was ineffective; characterization was achieved, however, by dideoxy sequencing using a tenfold increase in sequencing primer. The 39 base pair spacer was a filled-in *Eco*R1-*Pvu*II fragment of pAT153/*Pvu*II/8 (a pAT153 derivative constructed by Huddleston, Gould & Brownlee, unpublished), which was cloned into the appropriate vectors which had been previously restricted with *Bam*HI and treated with S1 nuclease and alkaline phosphatase. Positive recombinants were isolated by hybridization and dideoxy sequencing.

Transcription

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 analysed by electrophoresis on 6% polyacrylamide gels containing 7 M urea.

5′ end analysis of RNA *transcripts*

For each reaction, 3 ng of the mixture of the complementary strand box B oligos were labelled with 10 μCi [γ-^{32}P]ATP by using 0.3 units T4 polynucleotide kinase in 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 5 mM DTT for 1 h at 37 °C. The RNA products from four transcription reactions (see above) were used for the extension reaction, which was carried out at 42 °C for 1 h in a 10 μl volume containing the labelled primer, 50 mM Tris pH 8.3, 40 mM KCl, 8 mM $MgCl_2$, 10 mM DTT, all four deoxyribonucleotide triphosphates at 0.5 mM and 3 units reverse transcriptase. The products were fractionated on 16% polyacrylamide–7 M urea or 6% polyacrylamide–7 M urea gels before sequencing by the chemical degradation method (Maxam & Gilbert 1977).

Results

Analysis of box B *in a semisynthetic* RNA *polymerase* III *promoter*

The subclone used as a vector contains only the 5′ half of an Alu repeat promoter (box A and flanking sequences, see figure 1 line 2), and was previously demonstrated to be inactive as an RNA polymerase III template (see Paolella *et al.* 1983, and figure 2, plate 1). The mixture of double stranded box B-related oligonucleotides was cloned in the 'filled-in' *Eco*R1 site of clone 2 (figure 1), thus mimicking the naturally occurring relationship between box A and B. Seven out of the possible eight box B-related sequences were isolated (see figure 1); however, the 3′ G of the oligonucleotide GTTCTGTGAC consensus box was always deleted in this experiment (clone 4, figure 1). In addition, a wild type consensus box B (GTTCGAGAC) was isolated where the 3′ AG of the oligonucleotide was deleted (clone 10, figure 1).

These DNAs were tested as RNA polymerase III templates in the HeLa S100 *in vitro* system described by Weil *et al.* (1979) (figure 2). Lane 1 shows the transcripts produced by the parent Alu repeat cloned in pBR322 (Paolella *et al.* 1983), while lanes 2–10 show transcripts produced by the individual recombinants represented in figure 1 lines 2–10. Template activity was not detected either when box B was absent (figure 2, clone 2 lane 2) or when box B wild type sequence was present alone in the M13 vector (figure 2, lane 11). Promoter activity was restored

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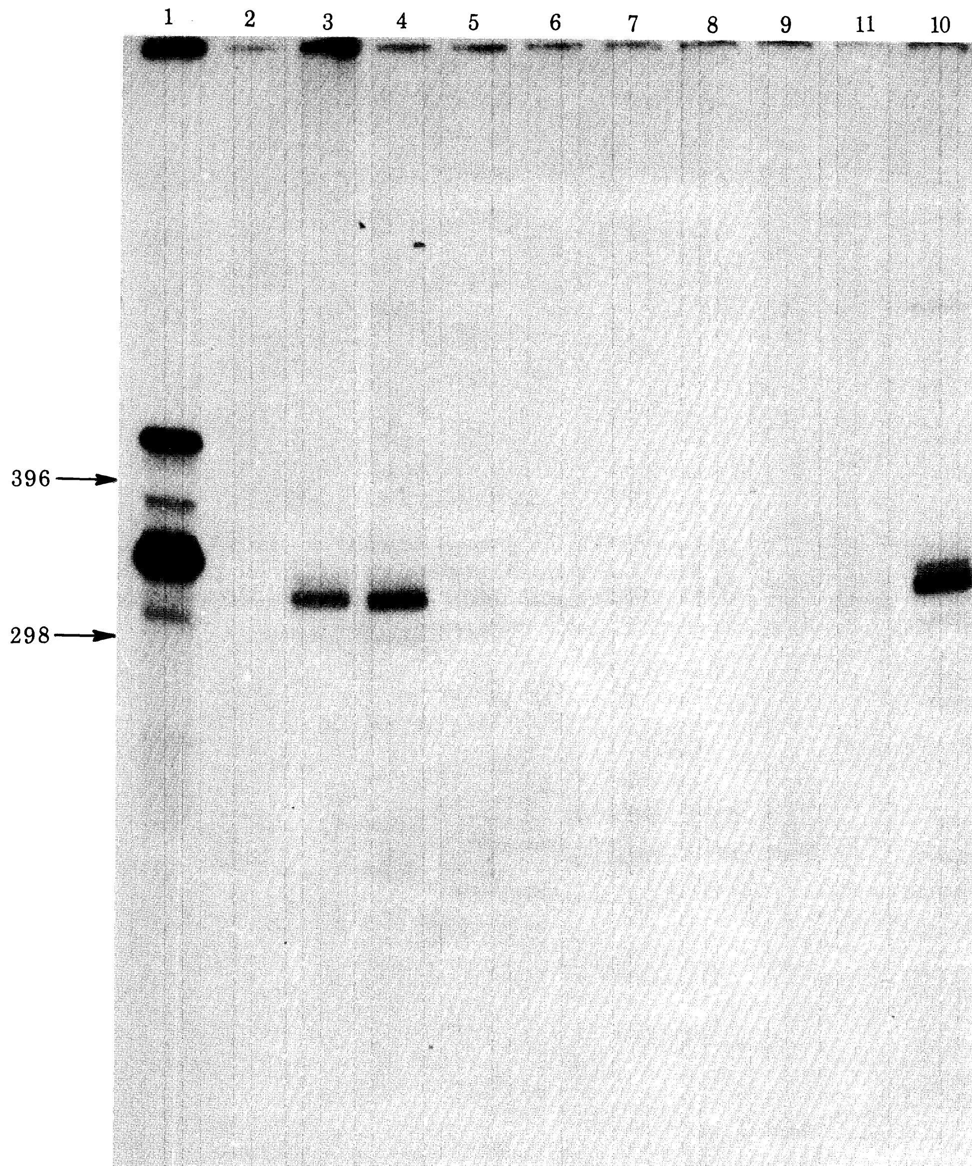

FIGURE 2. Autoradiograph of a 6% 7 M urea gel fractionation of RNA polymerase III transcripts from clones 1–11 (see figure 1). Single stranded size markers are indicated. The major transcript observed was approximately 330 nucleotides long, consistent with transcription initiation 5–15 nucleotides 5′ to box A as occurs in the natural Alu repeat, and termination at a strong stop in the M13 vector sequence.

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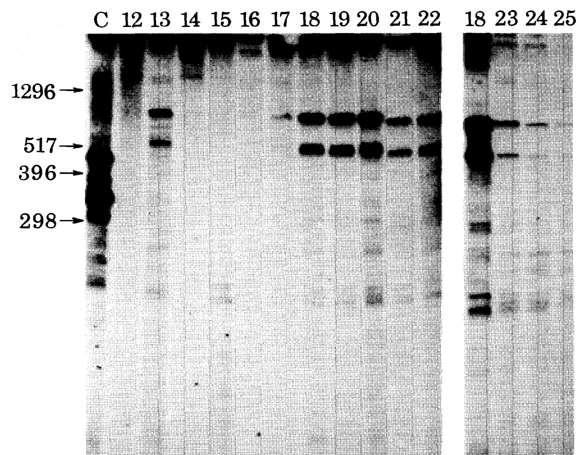

FIGURE 5. Autoradiograph of 6% polyacrylamide–7 M urea gel fractionation of RNA polymerase III transcripts from clones 12–25 (see figure 4). Lane C contains the products obtained by using a natural RNA polymerase III promoter as previously described (Paolella *et al.* 1983). The major transcripts terminate in M13 sequences (Paolella *et al.* 1983). DNA size markers in nucleotides are indicated.

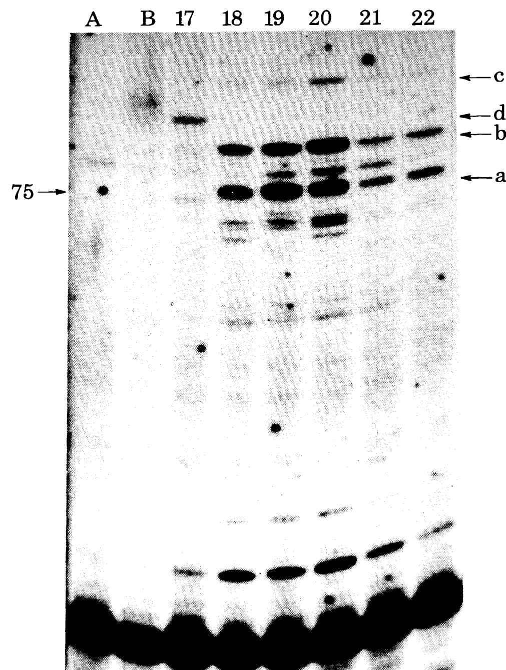

FIGURE 6. Autoradiograph of 16% polyacrylamide–7 M urea gel fractionation of primer extension products (see section on materials and methods). Lanes 17–22 are the 5′ end analyses of the RNAs synthesized by clones 17–22, respectively. Control lanes A and B differ from lane 18 in that clone 18 DNA is lacking in the former, while the HeLa extract was heat de-natured in the latter. Bands a–d are as described in results section. Bands a and b of clone 18 were sequenced by the method of Maxam & Gilbert (1977). DNA size markers in nucleotides are shown.

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in clone 3 where the chemically synthesized wild type box B was introduced into clone 2 (figure 2, lane 3). A similar activity was detected in clone 10 (figure 2, lane 10) where a wild type consensus sequence lacking the 3′ AG of the synthetic oligonucleotide is present. The major transcript observed was approximately 330 nucleotides long, consistent with transcription initiation 5–15 nucleotides 5′ to box A as occurs in the natural Alu repeat, and termination occurring at a strong stop in the M13 vector sequence (see Paolella *et al.* 1983). This was confirmed by using the *in vitro* produced RNA as template and the mixture of oligonucleotides complementary to box B as primer for cDNA synthesis with reverse transcriptase. A band 90 nucleotides long was observed which places initiation in the predicted region (data not shown).

An A→T transversion at the 6th position of the consensus sequence (figure 2, clone 4) similarly allowed promoter function. The additional nucleotide deletion at the 3′ end of the oligonucleotide in this clone is unlikely to be significant as it lies outside the consensus box; the equivalent activities of clones 2 and 10, which differ by a similar deletion, further indicate that a deletion at the 3′ end of the oligonucleotide does not affect transcription.

It is clear that a single G→C or T→A transversion at positions 1 and 3 respectively (clones 5 and 6) significantly reduces or abolishes transcription (see figure 2 lanes 5 and 6). In the case of multiple mutations, where two or three single mutations were introduced into the consensus B box (clones 7–9), no template activity was observed (see figure 2, lanes 7–9). This indicates that the G→C or the T→A transversions are dominant, and abolish the promoter function regardless of any other change in the consensus sequence (like the A→T transversion).

Construction of total synthetic RNA *polymerase* III *promoter*

The overall strategy for the construction of the synthetic RNA polymerase III promoter is illustrated in figure 3. A recombinant containing the wild type box B consensus sequence cloned in the *Hinc*II site of M13mp9 was selected as the primary precursor of the totally synthetic RNA polymerase III promoter (figure 3 and figure 4, clone 13).

The mixture of double stranded box A-related oligonucleotides was cloned into the *Sma*I site of this recombinant. All four possible sequence permutations were isolated and we also characterized a G_7→A transition which may have arisen from a by-product of the oligonucleotide synthesis. A 39 base pair long spacer was then cloned into the *Bam*HI restricted S1 nuclease-treated site of these clones to give the completed constructs (step 3). The sequences of the variant RNA polymerase III promoters and the precursors generated in their synthesis are shown in figure 4.

Individual DNAs were tested for activity as RNA polymerase III templates in the HeLa S100 *in vitro* system (Weil *et al.* 1979). Template activity was not detected when the wild-type box A oligonucleotide was present alone in the M13 vector (clone 12; figure 5, plate 2, lane 12), when the spacer was present alone (clone 15; figure 5, lane 15) and when box A and the spacer were cloned in tandem (clone 16; figure 5, lane 16). These results demonstrate that the box A and the spacer are not in themselves sufficient to form an active promoter. A low level of activity was apparent when only the box B wild type sequence was present in the vector (clone 13; figure 5, lane 13); however, this activity was reduced when the spacer was cloned downstream of the box B sequence (clone 17; figure 5, lane 17) and was totally abolished when the box A wild-type sequence was present immediately 5′ of the box B sequence (figure 3, step 2). The residual activity of clone 2 is therefore modulated by 5′ flanking sequences. Promoter activity was dramatically increased in clone 18 (figure 5, lane 18) in which all three

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FIGURE 4. Nucleotide sequence of the clones constructed in M13mp9. The DNA sequences corresponding to the RNA transcript are shown. The molecules read 5′ to 3′ from the left of the figure. M13 vector sequences are in lower case letters, the synthetic oligonucleotides in bold capital letters with variations from the wild type in underlined bold letters and the 39 nucleotide long spacer derived from the filled-in *Eco*R1–*Pvu*II polylinker of pAT/*Pvu*II/8 is in ordinary capital letters. H, B, E and S are the restriction sites *Hinc*II, *Bam*HI, *Eco*R1 and *Sma*I respectively. The recombinants tested as templates are: clone 12, wild-type box A alone; clone 13, wild-type box B alone; clone 15, the spacer alone; clone 16, wild-type box A and the spacer in tandem; clone 17, wild-type box B and the spacer in tandem; clones 18–22, the total constructs of the wild-type box B, spacer and box A, the variants that were generated are within the brackets and underlined; clones 22–25, the total constructs with a point mutant box B, spacer and box A variants. The sequence underlined is the box A-like region in the flanking M13. Arrows indicate the sites of initiation of clones 17–23 as determined by primer extension and Maxam & Gilbert sequencing.

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elements are present. The pattern of the major transcripts synthesized in the presence of this synthetic promoter is identical to that of a natural RNA polymerase III promoter cloned in M13mp9, in which leaky termination occurs as a result of sequences present in the vector DNA (Paolella *et al.* 1983). Point mutation variants of box A have no effect on promoter activity (clones 19 and 20; figure 5, lanes 19 and 20), although in clones containing double mutants there was a reduction in transcriptional activity (clones 21 and 22; figure 5, lanes 21 and 22).

The 5′ ends of the *in vitro* reaction products were analysed by primer extension by using the RNA as template and the 5′ ^{32}P-labelled mixture of oligonucleotides complementary to box B as primer for cDNA synthesis with reverse transcriptase. The major extension products when clone 18 was used to direct RNA synthesis were doublets of 80–81 nucleotides (figure 6, plate 2, band a) and 91–92 nucleotides (figure 6, band b) long, as indicated by Maxam & Gilbert sequencing of the ^{32}P cDNAs (data not shown). This places initiation of transcription at purine residues 7 or 18 base pairs 5′ to box A, which is consistent with the distance from box A observed in natural tRNA genes (Ciliberto *et al.* 1983). The longest extension product, though minor, was 114–115 nucleotides long (figure 6, band c). A further minor extension product of 98–99 nucleotides was observed when clone 17 which lacks the synthetic box A, was used as the promoter (figure 6, band d). In both these cases, transcription is initiating inefficiently at a position 14–17 nucleotides 5′ to a box A-like sequence which is present in the M13 vector (see figure 4). This would imply that the residual activity observed in clones 13 and 17, where a synthetic box A is not present, is being directed by a box A-like sequence in the vector. All the RNAs synthesized by the variant box A promoters (clones 19–22) were analysed by the primer extension technique and no qualitative differences were observed in the major primer extension products when compared with the wild type (figure 6, lanes 19–22).

The strategy employed in the construction of clones 18–22 allowed the excision of the box A and spacer sequences using *Eco*R1, and the subsequent cloning of these fragments into the *Bam*HI restricted S1 nuclease-treated site of an M13mp9 clone containing a mutant box B consensus sequence (clones 23, 24, 25). The A→T transversion present in box B has been shown previously to abolish template activity in a semisynthetic promoter (clone 6), and appears to be dominant in these constructs since only a residual activity was observed when they were tested as templates (figure 5, lanes 23, 24, 25).

Discussion

The construction of a synthetic promoter which mimics a natural RNA polymerase III promoter has allowed the essential elements required for efficient transcription *in vitro* to be defined accurately. The approach adopted enabled each individual component essential for transcription to be analysed either alone or in combinations. This is in contrast to other studies which have defined essential elements by assaying for an altered transcriptional activity in deletion or random mutants. The versatility of this synthetic technique is exemplified by the ability to create variations in a consensus sequence in a controlled fashion.

The results directly confirm for the first time the central role of the wild type box B consensus sequence in transcription as no template activity is observed when it is absent. Furthermore, two of the invariant nucleotides of the consensus sequence, G_1 and T_3, were shown for the first time to be essential for promoter activity (clones 5, 6 and 23). It was also shown that there is no absolute requirement of A_6 by box B for efficient transcription (clone 4) although this

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nucleotide is totally conserved in all functional tRNA and Alu promoters. It has been shown that the first step in the transcription of a VAI RNA gene by RNA polymerase III is the formation of a stable complex between a protein factor, TFIIIC, and box B. In the case of tRNA genes, this interaction is further stabilized by another factor, TFIIIB, to form a pre-initiation complex (Lassar *et al.* 1983). The residual activity observed in clones 5, 6 and 23, where point mutations render the template inactive, presumably reflects the impairment of this interaction. The inability of the box A variants (clones 24 and 25) to restore activity does not support models where tertiary interactions between boxes A and B are evoked (Hall *et al.* 1982). For example, these models would predict that a $G_{10} \rightarrow T$ transversion of box A may complement a $T_3 \rightarrow A$ transversion of box B to restore promoter activity. However, no significant difference between the residual template activity of clone 23 (wild type box A, $T_3 \rightarrow A$ box B) and clone 24 ($G_{10} \rightarrow T$ box A, $T_3 \rightarrow A$ box B) was observed.

The second element essential for efficient *in vitro* transcription is box A, the position of which relative to the 5′ end of box B is flexible. Box A is required for efficient transcription and defines the site of initiation of transcription 6–18 nucleotides upstream of the box A sequence. The greatly enhanced transcriptional activity and altered initiation pattern observed following insertion of a synthetic box A sequence clearly demonstrates this dual function (compare clones 17 and 18). The residual promoter activity of clones 13 and 17, which do not contain synthetic box A sequences, may be explained by RNA polymerase III and other protein factors interacting inefficiently with the pre-initiation complex and a box A-like sequence in the vector.

The introduction into the box A consensus sequence of either an $A \rightarrow G$ transition or a $G \rightarrow T$ transversion at the 6th or 10th positions respectively has no effect on promoter function (clones 19 and 20). A_6 and G_{10} are totally conserved in all tRNA genes, although G_6 is found in some functional RNA polymerase III Alu promoters (Ciliberto *et al.* 1983). As there is no absolute requirement for A_6 and G_{10} (or A_6 of box B) for efficient transcription, these nucleotides may play a functional role in the gene product or its processing; it has previously been demonstrated that a $G_5 \rightarrow T$ transversion in the tRNA box B consensus allows efficient transcription but only partial processing (Zasloff *et al.* 1982). Although the box A sequence is less stringently defined than that of box B, the reduction in transcriptional efficiency in clones 21 and 22 where there are two variations from the box A consensus sequence may reflect the cumulative departure from the wild-type sequence.

The results presented in this paper are in agreement with a general model proposed for tRNA promoters, in which the primary event seems to be the formation of a stable pre-initiation complex between protein factors and box B. Presumably RNA polymerase III and other protein factors interact with the pre-initiation complex and a 5′ box A sequence concurrent with initiation; it is not yet clear whether other sequences are involved in the modulation of transcriptional efficiency. The series of box A and B variants described here will help to further dissect these DNA-protein interactions.

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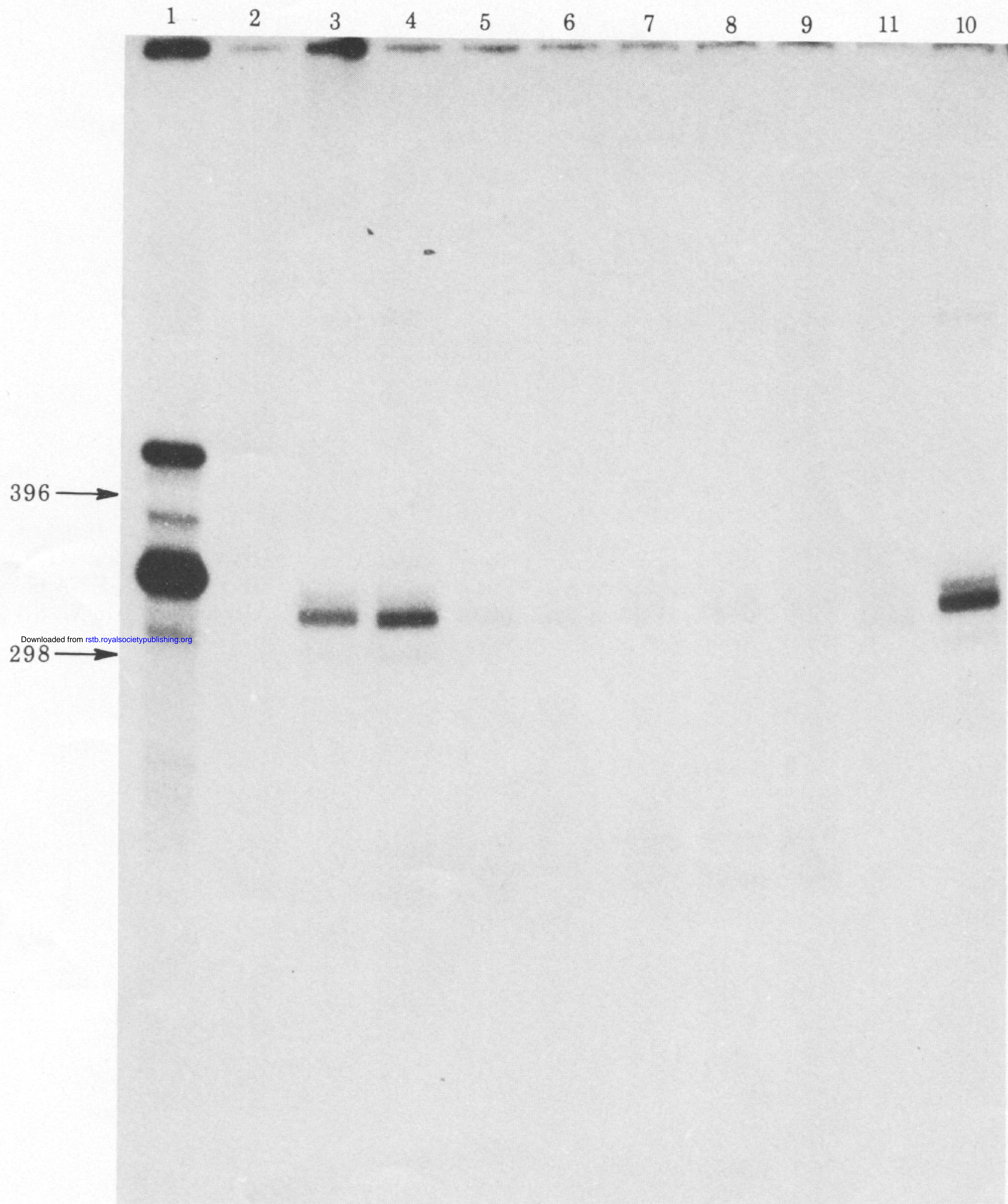

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FIGURE 2. Autoradiograph of a 6% 7 M urea gel fractionation of RNA polymerase III transcripts from clones 1–11 (see figure 1). Single stranded size markers are indicated. The major transcript observed was approximately 330 nucleotides long, consistent with transcription initiation 5–15 nucleotides 5′ to box A as occurs in the natural Alu repeat, and termination at a strong stop in the M13 vector sequence.

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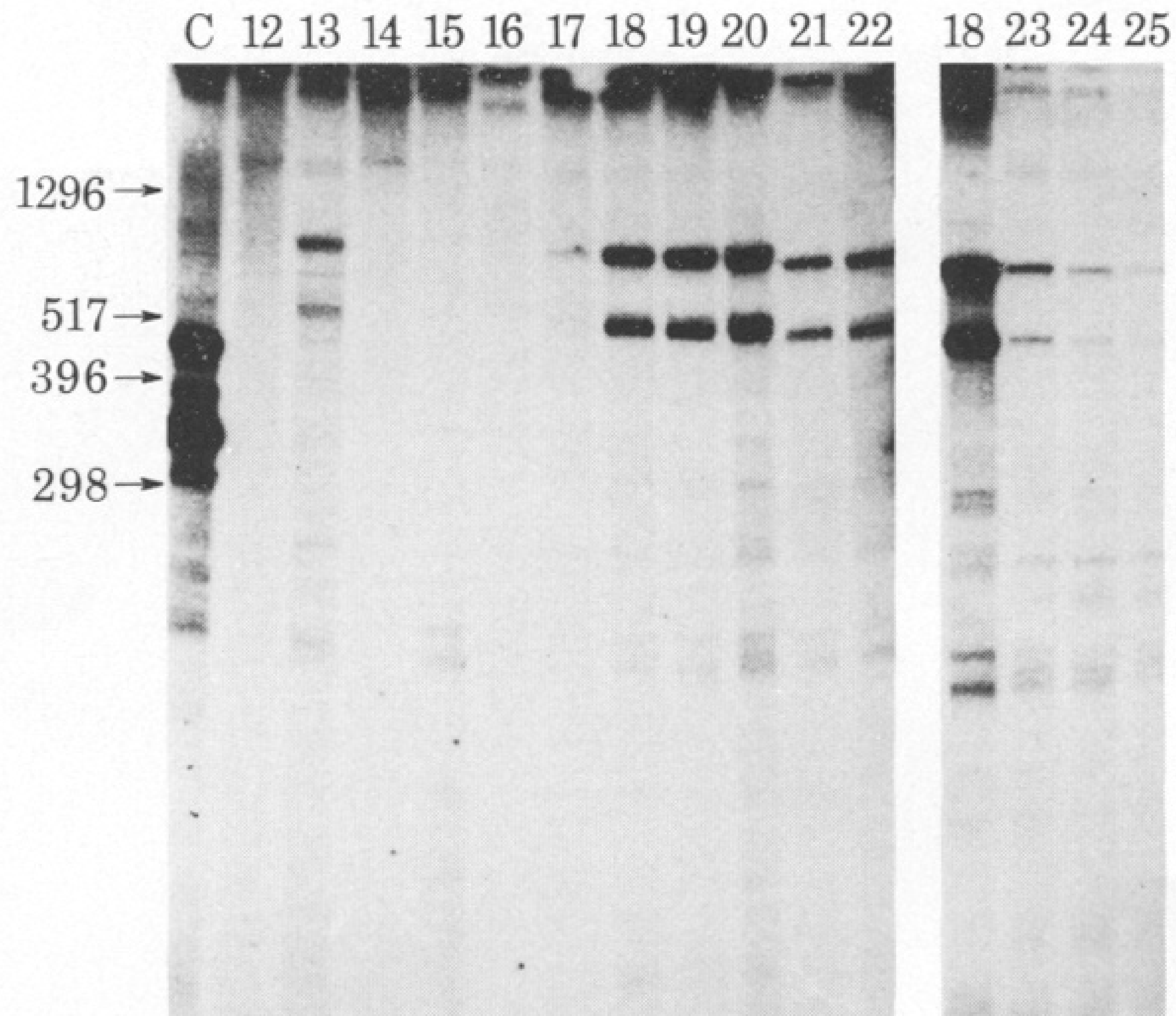

Figure 5. Autoradiograph of 6% polyacrylamide–7 M urea gel fractionation of RNA polymerase III transcripts from clones 12–25 (see figure 4). Lane C contains the products obtained by using a natural RNA polymerase III promoter as previously described (Paolella *et al.* 1983). The major transcripts terminate in M13 sequences (Paolella *et al.* 1983). DNA size markers in nucleotides are indicated.

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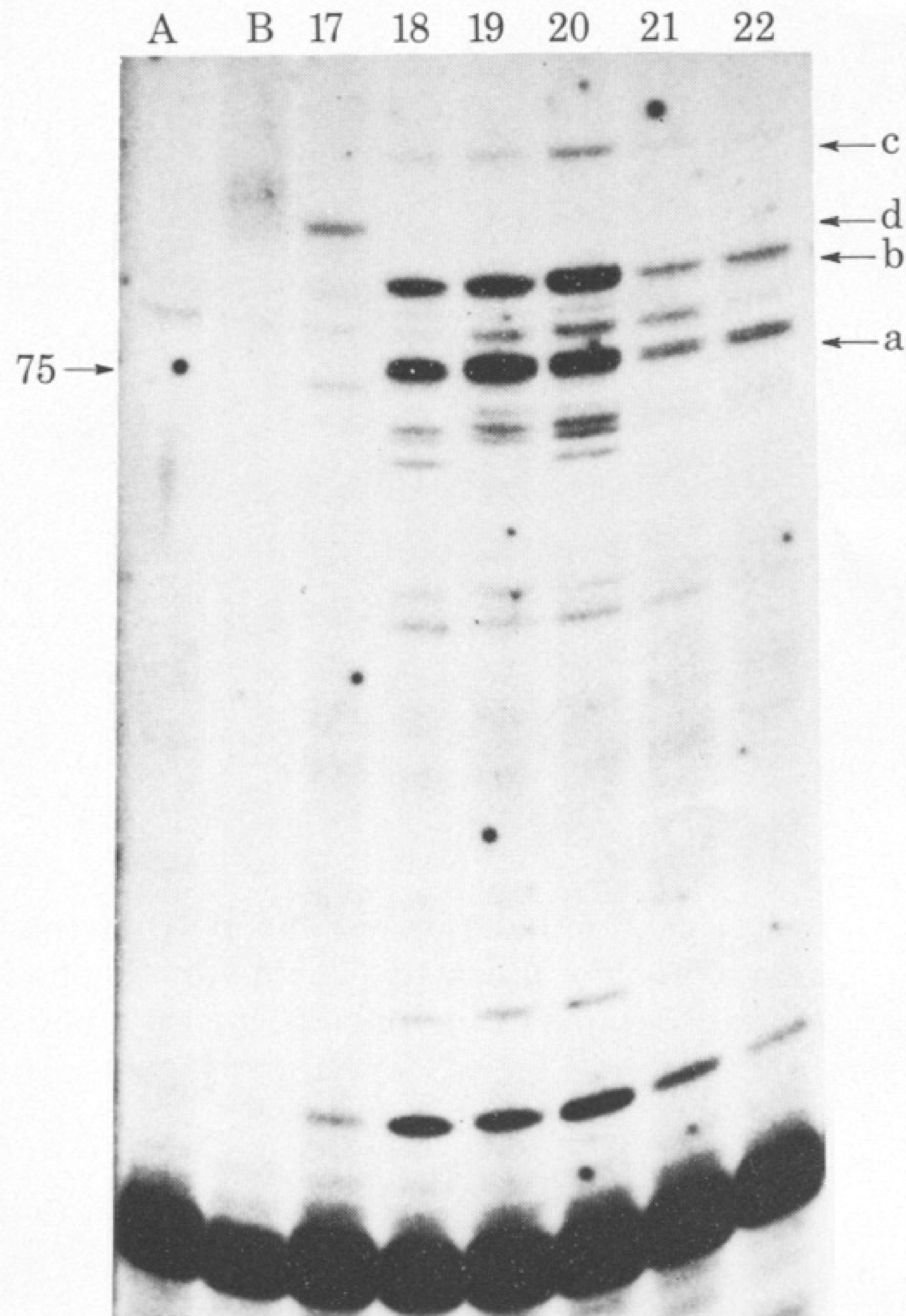

FIGURE 6. Autoradiograph of 16% polyacrylamide–7 M urea gel fractionation of primer extension products (see section on materials and methods). Lanes 17–22 are the 5′ end analyses of the RNAs synthesized by clones 17–22, respectively. Control lanes A and B differ from lane 18 in that clone 18 DNA is lacking in the former, while the HeLa extract was heat de-natured in the latter. Bands a–d are as described in results section. Bands a and b of clone 18 were sequenced by the method of Maxam & Gilbert (1977). DNA size markers in nucleotides are shown.