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Functions of and interactions between the A and B blocks in adenovirus type 2-specific *VARNA1* gene

(altered sequence and spacing/transcriptions/RNA polymerase III)

RONALD E. CANNON*, GUANG-JER WU†, AND JOHNNY F. RAILEY

Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, GA 30322

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ABSTRACT **The internal transcriptional control region (ITCR) of *VARNA1* gene consists of a 33-base-pair (bp) interblock sequence and two 12-bp sequence blocks that are highly conserved in most of the genes transcribed by RNA polymerase III. To define the functions of and study the interactions between the two blocks, we have constructed mutants with altered interblock sequence or spacing for transcription. The results of transcription efficiencies and competing strengths indicated that the interblock sequence was dispensable and the A and B blocks were essential for transcription control. One of the major functions of the interblock sequence was to maintain an optimal spacing for an intimate interaction between the two essential blocks. Shortening or elongating the interblock spacing in the mutants beyond this range drastically decreased the transcription efficiencies and competing strengths of these mutated genes. To further study how the interaction between the two blocks leads to initiation, the start sites and sizes of RNA products of the mutants were determined. When the interblock spacing was <105 bp, the wild-type start site was dictated by the A block after an interaction with the B block through proteins. However, when the interblock spacing was longer than 105 bp, several new start sites located closer to the B block were preferentially used. This suggests that new start sites may be dictated by the B block when its interaction with the A block is weakened by longer spacing. The mechanisms of interaction between the bipartite domain in this gene leading to initiation are different from those in tRNAs and *Alu*-family RNA genes.**

VARNA1 gene, one of the two small RNAs encoded by adenovirus (Ad), is synthesized in abundance in the late phase of the infected human KB cells (1–3). The gene is transcribed by the host DNA-dependent RNA polymerase III (4). Its transcription can also be mimicked actively, selectively, and accurately in a cell-free system derived from human KB cells (5, 6). This provides a convenient *in vitro* functional assay for determination of regulatory sequence and identification of regulatory factors necessary for transcriptional control of RNA polymerase III-mediated genes (7–9).

The essential control region of this gene is located intragenically in the coding sequence from +10 to +69 (7, 8). Within this internal transcriptional control region (ITCR), two 12 base-pair (bp) sequence blocks, centered at positions +18 and +63, respectively, are homologous to the two highly conserved sequence blocks in the two essential domains of the ITCR in tRNA genes (7, 10). *VARNA1* gene was, therefore, assumed to have a bipartite control region and mechanism similar to tRNA genes (10). To test this hypothesis, we have constructed mutants with altered sequence or spacing between the two blocks for transcription *in vitro*. We have quantitatively determined their transcription efficiencies and relative competing strengths. Furthermore, we have characterized the sizes and the start sites of their RNA products. We conclude that the *VARNA1* gene has a bipartite control region similar to tRNA genes, but the mechanism of interaction between the two blocks is different from that in tRNA genes. A preliminary account of this work has been presented (11).

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MATERIALS AND METHODS

Materials. [^{3}H]UTP (41–45 Ci/mmol; 1 Ci = 37 GBq), [α-^{32}P]UTP (350 Ci/mmol), [α-^{32}P]dATP (4500 Ci/mmol), and deoxyadenosine 5′-[α-[^{35}S]thio]triphosphate (650 Ci/mmol) were from Amersham. BAL31 nuclease, T4 DNA ligase, Klenow fragment of *Escherichia coli* DNA polymerase I, dideoxyribonucleotide DNA sequencing kit, and the pBR322 *Hin*dIII site primer for DNA sequencing were from New England Biolabs. *Kpn* I linker (dCGGTACCG) was from Collaborative Research (Waltham, MA).

Preparation of Cell-Free Extracts for *in Vitro* Transcription. Human KB cell-free extracts containing RNA polymerase III and necessary factors were prepared as described (5, 6) with minor modifications. The cytoplasmic S100 fraction was mixed with 0.5 vol of a cold solution containing 90% (vol/vol) glycerol, 30 mM Tris·HCl (pH 7.5), 120 mM KCl, 5 mM $MgCl_2$, and 7 mM 2-mercaptoethanol, and stored in 1- to 2-ml aliquots at −70°C.

***In Vitro* Transcription.** The reaction mixture in 50 μl contained 0.5 μCi of [α-^{32}P]UTP, either 0.5 μg of template DNA or 0.08–0.1 μg of template DNA and 0.4 μg of pBR322 DNA, and other necessary ingredients as described (5, 6).

Gel Electrophoresis of RNA and Autoradiography. RNA was purified (5, 6) and analyzed by electrophoresis in 8% polyacrylamide gels containing Tris/borate/EDTA buffer and 7 M urea (6). The gel was soaked in 10% methanol/10% acetic acid for 15–20 min to remove urea, dried, and exposed to x-ray film at −70°C with an intensifying screen.

Isolation of Covalently Closed Circular Plasmid DNA for Transcription. A large-scale method of preparation was used (12).

Construction of Mutated *VARNA1* Genes with Altered Interblock Sequence and Spacing. McKnight's scheme (13) of construction of linker-scanning mutants was adopted for construction of mutants with altered interblock sequence and mutants with altered interblock spacings (14). Two deletion mutation libraries were generated from a plasmid DNA containing intact *VARNA1* gene, pAdHinfC(ΔBB)R, at *Bam*HI and *Xba* I sites. Two appropriate members from the libraries were joined by ligation to form the mutants with

Abbreviations: ITCR, internal transcriptional control region; bp, base pair(s); Ad, adenovirus.

*Present address: Department of Genetics, North Carolina State University, Raleigh, NC 27695.
†To whom reprint requests should be addressed.

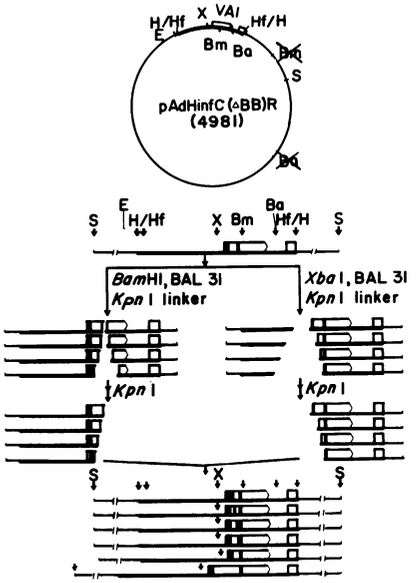

FIG. 1. Construction of mutated *VARNA1* genes with altered sequence and spacing between the A and B block consensus sequence. pAdHinfC(ΔBB)R (14) was used for generation of two deletion libraries. The heavy line stands for the inserted *Hin*fI fragment (from bp 10,069 to bp 10,685) of Ad type 2 (Ad2) DNA. The coding region of *VARNA1* and a portion of the coding region of *VARNA2* are indicated as open bars with and without arrow, respectively, which indicates the direction of transcription. The extent of deletion in each mutant plasmid DNA was determined by restriction analysis and verified by rapid DNA sequencing as described (15). Two appropriate members were joined by ligation to form mutants with altered interblock sequence and spacing (14). S, E, H, Hf, X, Bm, and Ba stand for *Sal* I, *Eco*RI, *Hin*dIII, *Hin*fI, *Xba* I, *Bam*HI, and *Bal* I restriction enzyme sites, respectively. The A and B blocks in the *VARNA1* gene are indicated as two closed zones. *Kpn* I linker is indicated as two vertical thin lines.

altered interblock sequence, all of the mutants with shorter interblock spacing, and some of the mutants with longer interblock spacing (Fig. 1). For some mutants with longer interblock spacing, a 45-bp foreign DNA fragment, which did not contain any potential terminator or attenuator, was chosen from the multiple cloning region of pUC19/18 plasmid (16) for insertion into the above mutants (see Fig. 4).

Determination of the 5′ Termini with the S1 Nuclease Mapping Method. A modified procedure of S1 nuclease mapping procedure was used (17, 18).

Determination of Relative Competing Strengths of the Mutants. Sequential transcription competition was done with an S100 extract in which free ribonucleoside triphosphates were removed by a G-75 column (19).

RESULTS

Transcription of Mutants with Altered Interblock Sequence. To define the function of the interblock sequence, six mutants with altered interblock sequence were tested for their transcription efficiencies and competing strengths for factors, as shown in Fig. 2. Alteration of the interblock sequence decreased at most about 50% of the relative transcription efficiency of the gene, suggesting that the interblock sequence is not essential for transcription control, but it may be needed for fine modulation. As also shown in Fig. 2, alteration of the interblock sequence had more substantial effect on relative competing strengths than on overall transcription efficiencies, suggesting that the interblock sequence from +40 to +55 is required to some degree for formation of stable preinitiation complexes.

Transcription of Mutants with Shorter Interblock Spacings. Having established that the interblock sequence is dispensable for essential transcriptional control, by elimination, the regions containing the A and B blocks ought to be the two essential functional domains for transcriptional control. To investigate the possibility that the major role of the interblock region is to maintain a proper spacing for an optimal interaction between the bipartite control domain, we constructed mutants with altered interblock spacing. Fig. 3 shows the DNA sequences of seven mutants with shorter interblock spacing. Shortening the interblock spacing drastically decreased the transcription efficiencies, as shown in Table 1. The minimal interblock spacing that rendered the gene still active for transcription, albeit with only 0.002 of the wild-type gene activity, was about 22 bp. Relative competing strengths of these mutants were also decreased in parallel to the transcription efficiencies (Table 1), indicating that shortening the interblock spacing also affects the formation of stable preinitiation complexes. As expected, the sizes of their RNA products were shortened in proportion to their interblock spacing, as shown in Figs. 5*A* and 6*C*.

Transcription of Mutants with Longer Interblock Spacings. To further study the effect of altered interblock spacing on

	A BLOCK +11 +21 +31 +41 +51	B BLOCK +61	BamH1 +71	TRANSCRIPTION EFFICIENCY (%)	COMPETING STRENGTH (%)
WT	CCGTGGTCTGGTGGATAAATTCGCAAGGGTATCATGGCGGACGACCGGGGTTCGAACCCCG			100	94
GWΔ7	CCGTGGTCTGGTGGATAAATTCCGCGGGGTATCATGGCGGACGACCGGGGTTCGAACCCCG			110	85
RC7N	CCGTGGTCTGGTGGATAAATTCGCAAGGGATACATGGCGGACGACCGGGGTTCGAACCCCG			78	52
RC8N	CCGTGGTCTGGTGGATAAATTCGCAAGGGCGGTACGGCGGACGACCGGGGTTCGAACCCCG			72	58
LSM1	CCGTGGTCTGGTGGATAAATTCGCAAGGGCGGTACCGCGGACGACCGGGGTTCGAACCCCG			82	50
LSM13	CCGTGGTCTGGTGGATAAATTCGCAAGGGTATCATGGCGCGGTACCGGGGTTCGAACCCCG			81	59
RC17O	CCGTGGTCTGGTGGATAAATTCGCAAGGGTATCATGGCGGCGGTACGGGGTTCGAACCCCG			58	44

FIG. 2. DNA sequences, transcription efficiencies, and competing strengths of wild-type and mutated *VARNA1* genes with altered interblock sequence. Only the DNA sequences from +11 to +71 including A and B blocks on the noncoding strand of the genes are shown. A and B blocks are shown by brackets. The altered sequences are shown by double underlines. ▽, *Bam*HI cleavage site. Relative transcription efficiencies of the mutants were determined by comparing to that of the wild-type gene (as 100%). Relative competing strengths were determined according to Schaack *et al.* (19). Total DNA concentration optimal for transcription was 0.25 μg/50 μl, which was brought up by pBR322 DNA. Plasmid with wild-type or mutated gene was used as a competing gene (0.1 μg). The reference gene (0.05 μg) used was a deletion mutant, JRB262, which was deleted from +72 to +105 and replaced by 8 bp of *Kpn* I linker; it was transcribed as efficiently as the wild-type gene and yielded an RNA smaller in size than the RNAs from the wild-type and mutated genes.

FIG. 3. DNA sequences of the first 101 nucleotides on the noncoding strand of seven mutated *VARNA1* genes with shorter interblock spacing. "+1" corresponds to the first nucleotide of the transcripts. A and B blocks are shown by brackets. The *Kpn* I linker is indicated as a double underline. The predicted sizes of 5'-labeled DNA fragments protected by RNAs synthesized from the wild-type and mutated genes from S1 nuclease digestion are shown below each DNA sequence. Δ, *Bam*HI site and the 5'-labeled end of the DNA fragment.

transcription and interaction between the two essential functional domains, mutants with longer interblock spacings were tested for transcription. The DNA sequences of 17 mutants with interblock spacings that varied from 34 to 239 bp are shown in Fig. 4. Elongating the interblock spacing from 34 to 60 bp did not significantly alter the transcription efficiencies, as shown in Table 1. However, elongating the interblock spacing longer than 60 bp drastically decreased the transcription efficiencies. The cutoff point of spacing was about 60–79 bp. Elongating the interblock spacing longer than 80 bp did not abolish the transcription efficiency; instead, these mutants still had a residual activity of about 2–5% of that of the wild-type gene. The effect was solely due to spacing rather than the sequence, as evidenced from the similar results obtained from the mutants with the insertion of multiple cloning fragment in either direction, such as RC1L and RC8L or RC31L and RC36L. The results of determination of the relative competing strengths of these mutants (Table 1) were in good agreement with the above results, indicating that a proper interblock spacing is required for formation of preinitiation complexes and efficient transcription.

Table 1. Transcription efficiencies and competing strengths of the wild-type and mutated genes with shorter and longer interblock spacing

Plasmid DNA	Active gene (*VARNA1*)	Interblock spacing, bp	Relative transcription efficiency,* %	Relative competing strength,† %
pAdHinfC(ΔBB)R	wt	33 (+0)	100	98
RC31	sm	29 (−4)	10	59
RC21	sm	29 (−4)	4	46
RC26	sm	27 (−6)	0.5	45
RC14	sm	22 (−11)	0.2	25
RC16	sm	18 (−15)	0	24
RC9	sm	17 (−16)	0	18
RC6	sm	15 (−18)	0	23
RC8	lm	34 (+1)	65	83
RC17	lm	34 (+1)	59	73
RC7	lm	37 (+4)	77	87
RC22	lm	37 (+4)	72	93
RC3	lm	43 (+10)	116	95
RC2	lm	49 (+16)	98	85
RC1	lm	59 (+26)	75	77
RC1L	lm	60 (+27)	91	77
RC8L	lm	60 (+27)	94	77
RC18L	lm	79 (+46)	31	68
RC28L	lm	88 (+55)	9	32
RC31L	lm	104 (+71)	3	35
RC36L	lm	104 (+71)	2.6	34
RC9L	lm	105 (+72)	3	29
RC31L2	lm	124 (+91)	2.5	26
RC17L	lm	149 (+116)	2.8	58
RC19L	lm	239 (+206)	0.8	46

wt, Wild type; sm, short mutant; lm, long mutant.

*One hundred percent is equal to incorporation of 1250 cpm of [α-^{32}P]UMP into the corresponding wild-type *VARNA1* resolved electrophoretically in gels. The results have been normalized for different UMP contents in the RNAs.

†One hundred percent is equal to incorporation of 1032 cpm of [α-^{32}P]UMP into the corresponding reference gene JRB262 RNA resolved electrophoretically in gels. Relative competing strength was determined and calculated according to Fig. 2.

As expected, the sizes of their RNA products were lengthened in proportion to their interblock spacings, except mutants RC17L and RC19L, as shown in Figs. 5*B* and 6 *A* and *B*. Surprisingly, mutant RC17L, with an interblock spacing of 149 bp, produced several unexpected shorter RNAs with apparent sizes slightly longer than or similar to the wild-type RNA as well as the expected longer RNA product (Fig. 5*B*, lane r). Furthermore, mutants RC17L and RC19L, with interblock spacings of 149 and 239 bp, respectively, produced a major RNA of about the same size as the wild-type RNA instead of the expected longer RNAs (Fig. 5*B*, lanes r and s). This suggests that alteration of interblock spacings may have effects on the use of start site of transcription.

The Start Sites of Transcription of the Mutants with Shorter and Longer Interblock Spacings. To investigate the possibility that the interblock spacing might have an effect on utilization of transcription start site, the S1 nuclease mapping method was used to determine the 5' termini of the RNA products from all of the mutants. The expected sizes of the 5'-end-labeled DNA fragments protected by RNA products were detected, if the mutants utilized the wild-type start site, except the mutants with interblock spacings longer than 149 bp, as shown in Fig. 6 *A–C*. A fragment size of 270 bp was not detectable for mutant RC19L; instead, a fragment size of about 80 bp was detected, as shown in Fig. 6*B*, lane g. Smaller, unexpected protected fragments were also detected from the mutants with a distance equal to or longer than 104 bp between the two blocks, such as RC31L, RC36L, RC9L, and RC17L, as shown in Fig. 6*B*, lanes c–f: this indicates that new start sites were chosen when the interblock spacing was equal to or longer than 104 bp. Fig. 6*C* shows that shortening the interblock spacing did not have any effect on the use of wild-type start site.

DISCUSSION

We have shown that the interblock sequence is not essential for an on/off transcriptional control (Fig. 2). Nevertheless, it is required for a fine modulation of transcription by providing

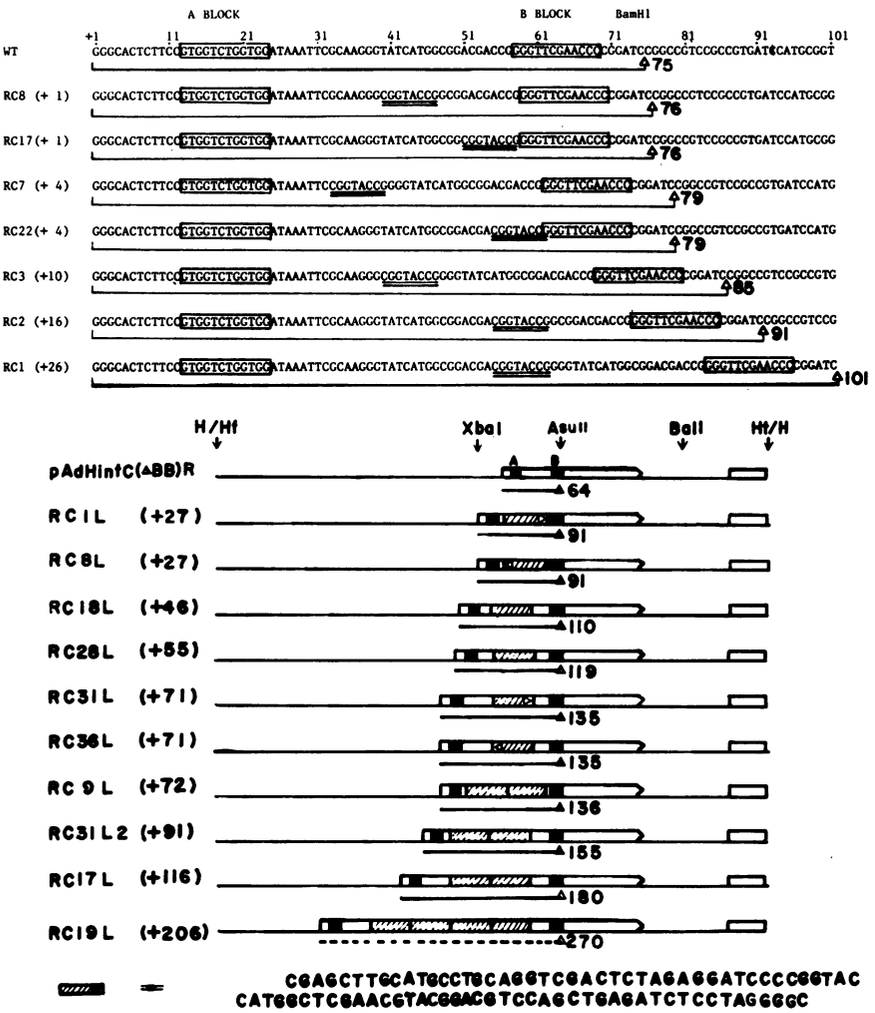

FIG. 4. DNA sequences of the noncoding strand of mutated *VARNA1* genes with longer interblock spacings and predicted S1 nuclease-resistant DNA fragment sizes. (*Upper*) DNA sequences of wild-type gene and seven mutants constructed as described in Fig. 1. (*Lower*) Genetic structures of mutants with longer interblock spacings constructed from an insertion of one or more than one copy of the 45-bp fragment derived from the multiple cloning region of pUC19/18. RC6 (−18), RC8 (+1), RC1 (+26), and RC3 (+10) were used to form RC1L, RC8L, and RC9L, RC18L and RC31L2, RC31L, RC36L, RC17L, and RC19L, and RC28L mutants, respectively. The fragment is indicated by brackets with slash lines. The orientation of the fragment is indicated by an arrow. Δ, The 5′-labeled end of the DNA fragment. The predicted sizes of S1 nuclease-resistant fragment are indicated by numbers next to the "Δ" sign.

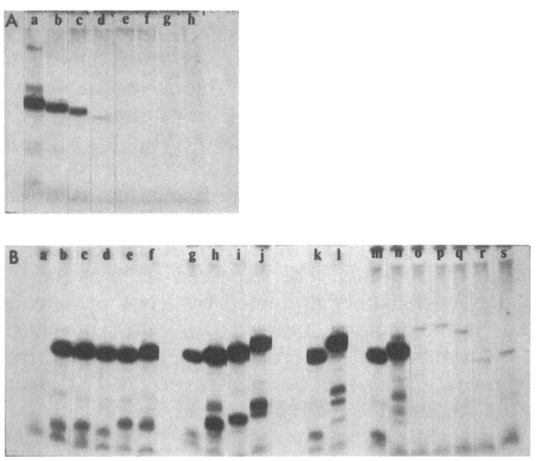

FIG. 5. Autoradiography of electrophoretically separated RNAs transcribed from the wild-type gene, mutated genes with shorter interblock spacing (*A*), and mutated genes with longer interblock spacing (*B*). In *A*, plasmid DNAs used for transcription were wild-type gene, pAdHinfC(ΔBB)R, and mutant RC17L genes (lane a), RC31 (−4) (lane b), RC21 (−4) (lane c), RC26 (−6) (lane d), RC14 (−11) (lane e), RC16 (−15) (lane f), RC9 (−16) (lane g), or RC6 (−18) (lane h). In *B*, plasmid DNAs used for transcription were pBR322 DNA (lane a), wild-type gene, pAdHinfC(ΔBB)R (lanes b, g, k, and m), RC7 (+4) (lane c), RC8 (+1) (lane d), RC17 (+1) (lane e), RC22 (+4) (lane f), RC3 (+10) (lane h), RC2 (+16) (lane i), RC1 (+26) (lane j), RC1L (+27) (lane l), RC8L (+27) (lane n), RC9L (+72) (lane o), RC31L (+71) (lane p), RC36L (+71) (lane q), RC17L (+116) (lane r), and RC19L (+206) (lane s).

the proper sequence for formation of stable preinitiation complexes (Fig. 2). This is consistent with the observation that the region from +46 to +83 is protected by the partially purified TFIIIC factors (20). The above result also suggests that the A and B blocks are the two essential functional domains within the ITCR.

Furthermore, we have proven that one of the major roles of the interblock region in transcriptional control is to maintain a proper spacing for an intimate interaction between the two domains. This allows formation of stable preinitiation complexes and efficient transcription (Table 1). Shortening the interblock spacing may create steric hindrance for the binding of regulatory factors and RNA polymerase III and, therefore, hamper the intimate interaction between the two domains. Elongating the interblock spacing seemed to be more tolerable than shortening the interblock spacing, suggesting that factors are able to bind to a large portion of the control region. Since one fraction of the factors, TFIIIC, can protect about 40 bp of the region flanking the B block sequence (20), binding of two fractions of factors should easily protect a region of at least 80 bp, which is close to the maximal control region of 84 bp (60 + 12 + 12 = 84) permitted for an intimate interaction between the two blocks. Longer interblock spacing seems to weaken this interaction and, therefore, decreases the overall transcription efficiency. The longest interblock spacing in which this interaction still can take place is between about 149 and 239 bp.

Analyses of the start sites of transcription of the mutants with altered spacing have provided insight into how the two domains interact and lead to initiation. When the interblock

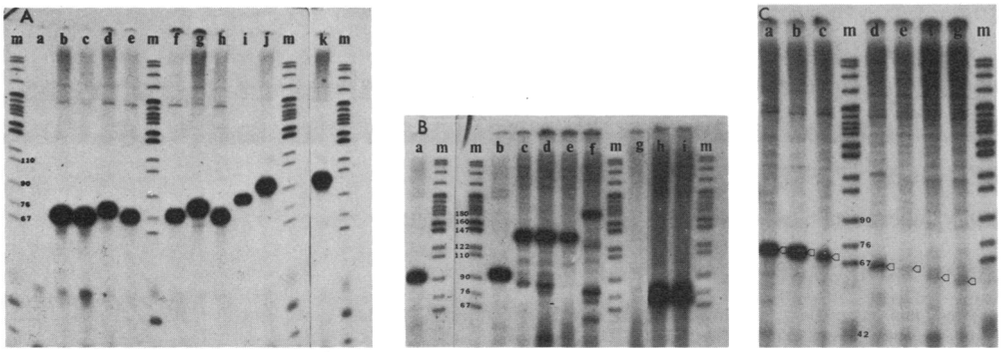

FIG. 6. Autoradiograph of S1 nuclease-resistant DNA fragments protected by RNAs synthesized from the wild-type and mutated *VARNA1* genes with longer interblock spacings (*A*) and (*B*) and with shorter interblock spacings (*C*). In *A*, RNAs were synthesized and DNA probes were derived from pBR322 DNA, wild-type gene (lane a), pAdHinfC(ΔBB)R (lane b), or pAdHinfC(ΔBB)L (lane c), mutated genes RC7 (lane d), RC8 (lanes e and h), RC17 (lane f), RC22 (lane g), RC3 (lane i), RC2 (lane j), and RC1 (lane k). In *B*, they were from RC1L (lane a), RC8L (lane b), RC31L (lane c), RC36L (lane d), RC9L (lane e), RC17L (lane f), RC19L (lane g), and wild-type gene pAdHinfC(ΔBB)R (lane h) or pAdHinfC(ΔBB)L (lane i). In *C*, they were from RC31 (lane a), RC21 (lane b), RC26 (lane c), RC14 (lane d), RC16 (lane e), RC9 (lane f), and RC6 (lane g). The 5′ ends of the DNA fragments of the wild-type gene and the mutants with and without the multiple cloning region fragment were phosphorylated with [γ-^{32}P]ATP and T4 polynucleotide kinase after linearization with *Asu* II and *Bam*HI, respectively. [^{3}H]RNA synthesized *in vitro* (5, 6) was mixed with the corresponding 5′-end-labeled DNA fragment and hybridized at 63°C for 16 hr. The hybrids were digested with S1 nuclease, denatured in 96% formamide, and electrophoretically analyzed. ^{32}P-labeled *Hpa* II-cleaved pBR322 DNAs (lane m) were used as size standards. The protected fragments are shown as arrows in *C*.

spacing was <105 bp, the start site was always located at a fixed distance upstream of the A block sequence, suggesting that A block is involved in dictating the start site. But when the interblock spacing was >105 bp, several new start sites located in the interblock region were utilized; therefore, the unexpected shorter RNAs were produced (Figs. 5 and 6). When the interblock spacing was equal to 239 bp, the wild-type start site was not used; rather, a new start site closer to the B block was chosen. This major new start site is located about 72 bp upstream of the B block. This suggests that when the interblock spacing is longer than 105 bp, the interaction between the two blocks is so weak that the B block sequence may dictate new start site(s) closer to it.

An alternative explanation for the use of new start site(s) may be attributed to introducing into the gene by the foreign DNA fragment a weak A block-like sequence that cooperates with the strong B block to direct transcription. After careful searching of the sequence in the fragment of the multiple cloning region, we conclude that it is highly unlikely that there is a functional weak A block-like sequence in this DNA fragment.

When the B block was moved relative to the A block, it was also moved, at the same time, with respect to the 5′ flanking sequence, which has been shown to contain sequence required for the use of start sites and efficient transcription (refs. 7 and 21; unpublished results). Our results presented here of mapping the start site indicated that the wild-type start site was always used when the interblock spacings of the mutants, which had the same 5′ flanking and A block sequence, varied from 15 to 105 bp, suggesting that the contribution of 5′ flanking sequence to transcription control and use of start site is a common denominator.

We have shown that parts of the functions of the two essential domains in the *VARNA1* gene are similar to those in an *Alu*-family RNA gene (22) and tRNA genes (23–25); however, the interaction between the two essential functional domains in the *VARNA1* gene appears to be more intimate than those in tRNA genes and the *Alu*-family RNA gene. This is corroborated by the finding that the *VARNA1* gene is transcribed 10–20 times more efficiently than both classes of genes (unpublished results). Furthermore, studies on interactions of factors with these genes indicate that two fractions, TFIIIB and TFIIIC, are required for formation of stable preinitiation complexes with tRNA genes, whereas only one fraction of factors, TFIIIC, is required for formation of stable preinitiation complexes with *VARNA1* gene (9). This further supports the notion that the mechanism of interactions between the two essential functional domains in the *VARNA1* gene is different from that in vertebrate tRNA genes.

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