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# Large numbers of random point and cluster mutations within the adenovirus VA I gene allow characterization of sequences required for efficient transcription

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ABSTRACT

We have isolated clones with well over 100 randomly dispersed point mutations distributed throughout the 5' half of chemically synthesized adenovirus type 2 VA I genes. In addition, we have isolated clusters of mutations targeted to the regions corresponding to the A and B block consensus sequences of eukaryotic tRNA and adenovirus VA genes. *In vitro* analyses of these constructs have allowed us to survey in detail the importance of DNA sequence to transcriptional efficiency. Our analyses demonstrate that certain constructs with radically substituted A block regions can be transcribed efficiently. In contrast, there is little tolerance for variation in the sequence within the B block region. We propose that the B block sequence should be R-G-A/T-T-C-R-A-N-N-C for optimal transcriptional efficiency of the VA I gene in mammalian cells.

## INTRODUCTION

The VA I (virus-associated) RNA of adenovirus type 2 (Ad-2) is one of two small (160 nucleotides) virally encoded RNA polymerase III products produced abundantly in cells during the late phase of infection (1, 2). Its function has recently been linked to the efficient translation of viral mRNA. VA I blocks the activation of a cellular kinase that phosphorylates and inactivates translation factor eIF-2$\alpha$ (3, 4).

Transcription of VA I is similar to other genes transcribed by RNA polymerase III such as 5S RNA and tRNAs. VA I, tRNA and 5S RNA all require polymerase III transcription factors B and C. In contrast, the 5S gene requires an additional component, factor A (5). The intragenic promoter, which is common to VA I and tRNAs, consists of an A and a B block separated by an interblock spacer as shown in Fig. 1A (6, 7). The 5S gene is somewhat different in that it has a defined A block but seems to lack or have a more cryptic B block (5). Most of the functional analyses of these genes have been carried out using cell free RNA polymerase III transcription systems that allow for the rapid investigation of transcription factor and sequence requirements (5, 6, 7, 8, 9).

Until the recent development of inexpensive oligonucleotide synthesis, the capability of detailed DNA structure-function analysis through the generation of altered sequence was limited. Deletion mutagenesis, which removes large blocks of sequence, is useful but defines promoter regions rather crudely. This technique also adds the possibility of generating artifacts

when previously separated DNA sequences are brought together (6, 10). Chemical mutagenesis (11) utilizes compounds such as sodium bisulfite to produce C to T transitions but these mutations are limited in scope. Because oligonucleotide directed site specific mutagenesis (12) only allows mutation of a few nucleotides at a time, it is impractical for producing large numbers and varieties of mutations. In addition, this technique suffers from the selective prejudices of the researcher. None of these methods can be used to quickly and easily generate a variety of mutations at all sites within a given target sequence while leaving surrounding wildtype sequences intact.

We utilized a more efficient method to generate libraries of VA I genes containing either randomly dispersed (13) or clustered point mutations. Mutations were generated during oligonucleotide synthesis by deliberately contaminating each nucleotide phosphoramidite with low levels (2.5%) of other phosphoramidites. Complementary oligonucleotides were synthesized with overlapping sticky ends and assembled by thermal denaturation and slow-cooling to anneal the oligonucleotides in proper sequence. The assembled fragments contained convenient restriction sites for shotgun cloning into an M13 vector to produce a library of mutant VAI genes. Clones were sequenced to determine the locations and types of mutations they contained, double-stranded DNA was prepared and *in vitro* transcription assays were used to assess the effect of a given mutation on transcription efficiency.

Initially, we constructed clones with mutations distributed throughout a 100 bp region from the upstream XbaI site to the mid-gene BamHI site (Fig. 1). We subsequently constructed clones with mutations confined to sequences contained within and immediately surrounding the A and B blocks. In this case, oligonucleotides were synthesized to randomly change on average one half of the targeted sequences in each clone to transversion mutations. Our transcriptional analyses of the VA I mutants indicate that certain previously uncharacterized single base changes have dramatic effects. The results of these experiments add to our knowledge of the sequence requirements of the VA I promoter.

## MATERIALS AND METHODS

### Chemical Synthesis of DNA

Oligonucleotides VA I.1 through VA I.9 were synthesized using o-methyl phosphoramidite chemistry, while VA I.10 through VA I.14 were synthesized using $\beta$-cyano-ethyl phosphoramidite chemistry (14). The cloning efficiency of oligonucleotide constructs synthesized by each of these methods was similar. Oligonucleotides were synthesized using an Applied Biosystems 380A DNA synthesizer. Phosphoramidites were obtained from American Bionetics and Applied Biosystems. Libraries of mutant VA I genes were constructed from the synthetic oligonucleotides shown in Figs. 1 and 2. The four oligonucleotides composing the 3' end of the VA I gene (VA I.4, VA I.5, VA I.8, and VA I.9) were synthesized using uncontaminated phosphoramidites. Thus these oligonucleotides were identical in sequence to the

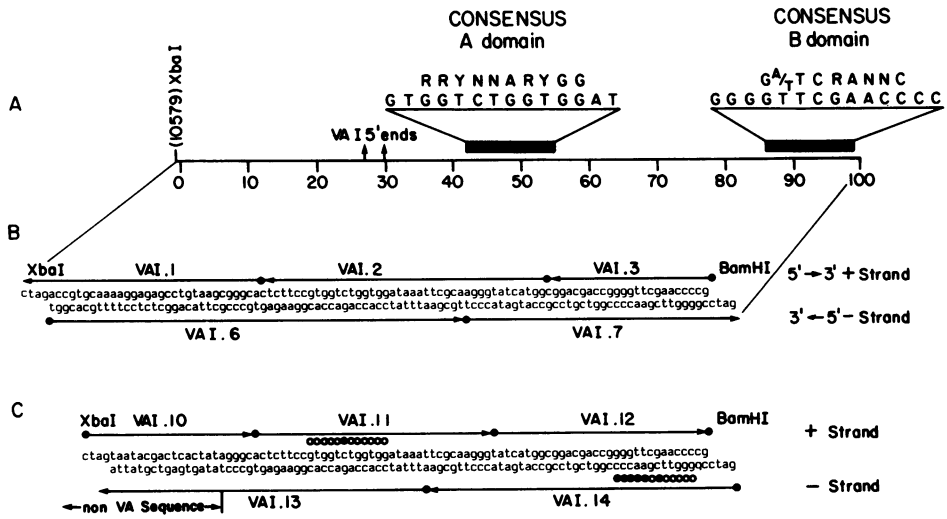


Figure 1: Diagram of the two types of VA I 5' ends assembled from oligonucleotides. (A) The VA I gene showing: 5' transcriptional start sites and the A and B domain plus consensus sequence (17, 23). The XbaI and BamHI cloning sites are also shown. The number in parentheses at the 5' terminus represents the nucleotide number from the Ad-2 genomic sequence according to Genbank, May, 1985. (B) The oligonucleotides used to assemble the 5' end of the m232 random point mutation series (VAI, .1, .2, .3, .6, .7) showing restriction sites and strand orientation. (C) The oligonucleotides used to assemble the 5' end of the m241-242 series. This fragment differs from the m232 series in having non-VA sequence from position 21 to the XbaI site. Also, the mutations generated were restricted to the plus strand for the A block and minus strand for the B block. Mutated sequences are denoted by an open circle for 1:1 T+G synthesis and a filled circle for 1:1 A+C synthesis.

wildtype Ad-2 VA I gene (Fig. 2). Generation of the mutant oligonucleotides comprising the 5' half of the construct followed the method of Hutchison *et al* (15). The five oligonucleotides used to assemble the 5' end of the m232 series library (for random point mutations) were mutated during synthesis by adding 200 μl of an equimolar mixture of the four phosphoramidites to 6 mls of the pure phosphoramidites used in the synthesis shown in Fig. 1B.

Mutations in the m241-242 series library (for clustered mutations) were limited to the region of the A block in the plus strand and the region of the B block in the minus strand. At the positions indicated in Fig. 1C corresponding to an A or C in the wildtype sequence, a 1:1 mixture of A and C phosphoramidites was used in the synthesis of the library. Conversely, a 1:1 mixture of T and G was used during synthesis at positions corresponding to a wildtype T or G. In the m241-242 library, the sequences upstream of the VA I transcription initiation site were replaced with sequences unrelated to the normal Ad-2 sequences.

It had been noted previously that the wild type VAI gene terminates inefficiently (6). In an attempt to improve termination efficiency, we increased the stretch of thymidines at the 3' end

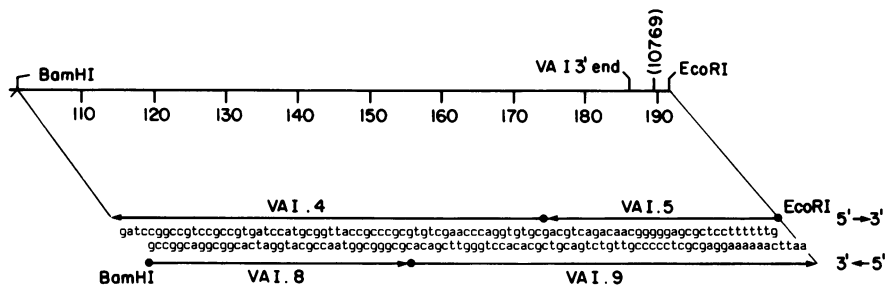


Figure 2: The wildtype 3' end of the VA I gene used in all libraries, assembled from oligonucleotides VAI.4, .5, .8, .9. Also shown are the transcription termination site and the BamHI and EcoRI cloning sites. The number in parentheses at the 3' end of the gene represents the number from the Ad-2 genomic sequence according to Genbank, May, 1985.

of the gene from 4 to 6. This resulted in nearly 100% termination at the 3' end of the gene in our transcription reactions.

Cloning

All oligonucleotides were purified by polyacrylamide gel electrophoresis. Oligonucleotides with 5' ends internal to each construct were phosphorylated. The kinase was heat inactivated, and oligonucleotides were mixed in equimolar ratios, annealed by heating to 65°C followed by slow cooling to room temperature, and then ligated overnight at 14°C. Ligated constructs were purified by polyacrylamide gel electrophoresis. The synthetic VA I genes were cloned into a modified M13 mp18 vector as shown in Fig. 3. This vector, designated mp18.1, has several restriction sites inserted between the BamHI and XbaI sites of the mp18 polylinker. The oligonucleotides used to construct the VA I genes were assembled into 5' and 3' fragments and cloned into mp18.1 in two steps. The four oligonucleotides composing the 3' end of the gene were assembled and ligated into mp18.1 cut with BamHI and EcoRI. The ligation mixture was used to transform JM107 (15), and individual plaques were selected and sequenced by the dideoxy chain termination method (16) to verify insertion of the 3' end of the VA I gene. Phage replicative form (RF) was prepared (17), cut with BamHI and XbaI, and ligated with the assembled oligonucleotides comprising the 5' half of the VA gene. The ligation mixture, cut with ApaI, SstI and XhoI to reduce background, was used to transform JM101. The transformation mix was plated and incubated overnight at 37°C. Plates were overlayed with SM buffer (18) for 1 hour. The buffer was removed, centrifuged and the supernatant pasteurized at 65°C for 15 minutes and stored at 4°C. These phage stocks were diluted and plaqued. Single stranded DNA for sequencing was prepared by standard methods (19). The cell pellets from these preparations were pasteurized and stored at -20°C for use as seed stocks.

RF Preparation

Phage were plated in top agar (18) on LB plates. Plaques were picked with a sterile

toothpick and transferred to 1 ml of L-broth containing 2 μl of an overnight JM101 culture. Cultures were grown for 7 hours at 37°C with shaking and then centrifuged for 5 minutes in a microfuge to pellet the bacteria. The supernatant was transferred to a new tube and pasteurized 15 minutes at 60°C. Pasteurized supernatant plus 2 ml of overnight JM101 were added to 1 liter of L-broth and grown overnight at 37°C in a shaking incubator. RF DNA was then prepared according to the procedure of Garger *et al* (17).

Extracts and Transcription Reactions

Cell extracts containing RNA polymerase III activity were prepared as described by Weil *et al* (9) from spinner cultures of KB cells. Transcription reactions and analyses of RNA transcripts were carried out under standard conditions as previously reported (6). All reactions contained 0.4 μg RF DNA in a 50 μl reaction volume. $^{32}P$ labeled transcripts were electrophoresed under denaturing conditions on a 6% polyacrylamide gel. The gels were dried and autoradiographed; bands containing transcripts were cut out, and Cerenkov counts were measured in a scintillation counter. Transcription levels, measured as percentage of wildtype, were calculated by comparing radioactivity in transcript bands from mutant templates with that in the band from a wildtype template. All results were verified by using two or more independent preparations of transcription extracts. To assess the fidelity of our results, transcription reactions were also carried out in the presence of a mini-VA gene, pA2-dl1, as previously described (6). This allowed transcription from mutant templates to be compared to that from an internal standard.

## RESULTS

The library of mutant VA I genes was assembled and cloned as shown in Fig. 3. The 3' half was assembled from oligonucleotides and inserted into a modified M13 vector, mp18.1, giving rise to a clone called M13mp3'VA. Subsequently, the 5' end of the VA I gene was assembled from randomly mutated oligonucleotides and cloned into M13mp3'VA to generate a library with a large population of mutant clones. Single stranded templates from numerous isolates were prepared for rapid sequencing by the chain termination method (16). Mutants from the m232 library were categorized as being 5' nonblock, interblock, or either A or B block mutants.

The combined distribution of all sequenced point mutations in the m232 library is shown in Fig. 4. Contamination of pure reagents with a 1:30 ratio of the mutating phosphoramidites resulted in an average of 2.5 mutations per VA I gene. While providing good coverage of the entire area of interest, this ratio also produced a sufficiently low mutation frequency to allow the analysis of numerous isolates with only one or two point mutations. A total of 48 nucleotides were mutated to A, 26 to C, 16 to G, and 56 to T. It seems likely that the high proportion of changes to A or T is due to an inadvertent bias in the concentrations of contaminating phosphoramidites. Because both strands of the synthetic gene were mutated, a

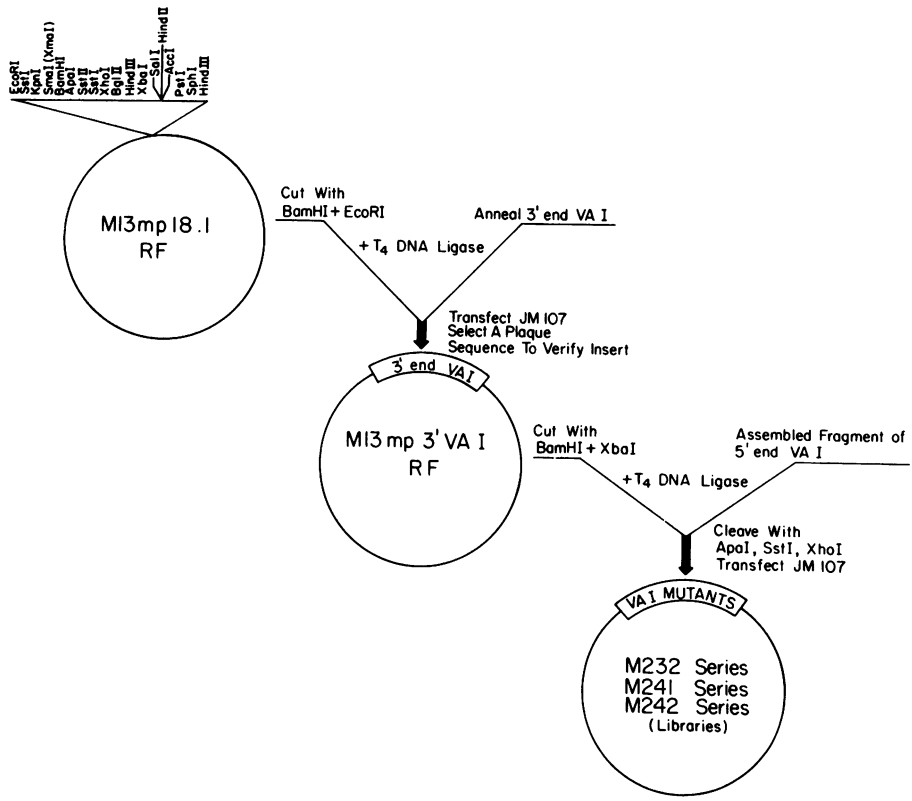


Figure 3: Library construction. The flow diagram shows the cloning strategy used to produce the m232, 241 and 242 series libraries. Cleavage of the ligation mix containing M13mp3'VAI plus annealed 5' VA I end fragment with ApaI, SstI and XhoI was carried out to reduce the background of parental clones.

disproportionately high concentration of the contaminating A or T phosphoramidite would explain the observed results.

The m241 and m242 isolates reported in this study contained multiple mutations confined solely to the A or B block region. Isolates with mutations in the A block region outnumbered those with B block mutations by a ratio of 3:1. This probably represents the known strand replication bias of M13 (19). Several isolates in these series had wildtype A and B blocks; however, no isolates examined had both A and B block mutations.

Non A or B Block Mutations

Point mutations in the 5' and interblock VA regions do not alter *in vitro* transcription to an extent which would implicate these sequences in promoter function. This supports published point mutation data which suggest that interblock regions have little or no role in VA I transcription (see Discussion). However, multiple mutations occurring near the B block depress


Figure 4: Point mutations. The distribution of all mutations sequenced from the random point mutagenesis m232 series is aligned beneath the mutated sequences.

transcription somewhat (20). In the construction of our mutant libraries, multiple clustered mutations were not generated in the interblock region. Thus the effects of multiple changes near the A or B blocks cannot be assessed.

A Block Mutations

Mutations generated in the A block and their effects on *in vitro* transcription are shown in Fig. 5. Sequences outside of the A block remained wildtype. Mutations at two positions

| | Position # / Consensus / Wildtype | VAI A Block Region Point Mutations | Transcriptional Signal % Of Wildtype |
|---|---|---|---|
| | | -1 1 2 3 4 5 6 7 8 9 10 | |
| | | R R Y N N A R Y G G | |
| | | T T C C G T G G T C T G G T G G A T | |
| A | m232- 46 | A | 100 |
| | 67 | T | 100 |
| | 90 | A | 60 |
| | 1 | A | 40 |
| | 44 | A | 40 |
| | 42 | A T | 20 |
| | 101 | C | 40 |
| | 47 | A T | 60 |
| | 27 | A | 60 |
| | 13 | T | 60 |
| | 84 | A | 80 |
| | 54 | C | 80 |
| | 4 | G | 60 |
| | 88 | del | 100 |
| B | | VAI A Block Region Clustered Mutations | |
| | m241- 5 | T G T T G T | 40 |
| | m242- 1 | G T G G T T | 40 |
| | m241- 28 | T G A G T G | 60 |
| | m241- 2 | G T G G T G | 80 |
| | m241- 47 | G G A G T G T T | 80 |
| | m242- 2 | G T T G G | 80 |
| | m241- 30 | G G T G T | 90 |

Figure 5: Effect of A block mutations on transcription. (A) The wildtype sequence, the A block consensus sequence (17), and numbers of consensus sequence positions are given at the top of the diagram. Names of mutants are given at the left and transcriptional activities of mutants relative to a wildtype control are given at the right. Mutants which show identical point mutations in the A block (m232-1, m232-44) possess different mutations in nonblock regions. (B) The clustered mutation data derived from the m241 and m242 libraries are presented as in A.

| | Position # / Consensus / Wildtype | | VAI B Block Region Point Mutations<br>-2 -1 1 2 3 4 5 6 7 8 9<br>G A/T C R A N N C<br>A C C G G G G T T C G A A C C C C | Transcriptional Signal % Of Wildtype |
|---|---|---|---|---|
| A | m232- | 82 | T | 90 |
| | | 81 | del | 80 |
| | | 44 | A | 30 |
| | | 65 | A | 30 |
| | | 26 | A | 80 |
| | | 75 | A | 90 |
| | | 85 | T | 10 |
| | | 102 | C | 20 |
| | | 5 | A | 80 |
| | | 9 | A | 60 |
| | | 36 | A | 80 |
| | | 6 | T | 80 |
| | | 47 | T | 60 |
| | | 23 | G | 80 |
| | | 35 | T | 80 |
| | | 51 | T | 90 |
| B | | | VAIB Block Region Clustered Mutations | |
| | m242 - | 4 | G C A A A | 10 |
| | | 6 | G G A T C C A | 10 |
| | m241 - | 7 | T T A | <10 |
| | | 36 | T G A A A | <10 |
| | | 37 | T G G A T C A | 10 |
| | | 66 | T T A | <10 |

Figure 6: Effect of B block mutations on transcription. (A) The wildtype sequence, the A block consensus sequence (23), and the numbers of consensus sequence positions are given at the top of the diagram. Names of mutants are given at the left and transcriptional activities of mutants relative to a wildtype control are given at the right. Mutants which show identical point mutations in the B block (m232-5, m232-36) possess different mutations in nonblock regions. (B) The clustered mutation data derived from the m241 and m242 libraries are presented as in A.

decreased transcription to less then 50% of wildtype. Transversion mutations of the T at position -1 or G at position 1 of the A block consensus sequence caused a decrease in transcription to 40% or less of wild type. (Fig. 5A, m232-1, -42, -44 and -101).

Interestingly, clustered mutagenesis of the A block (Fig. 5B) did not significantly decrease transcription. It is especially noteworthy that mutants m241-2, m241-47 and m242-2, which have transversions at postition -1, 1 or at both positions, show 80% of wildtype transcription. In contrast, transcription is decreased to less than 50% of wildtype in mutants m232-1, -44, -42, and -101, which have mutations restricted to position 1, -1, or both positions (Fig. 5A, mutants m232-1, -44, -42, and -101). This suggests that T to G transversions may be less deleterious than T to A transversions at position -1.

B Block Mutations

The point mutations surveyed within and around the B block are shown in Fig. 6A. We found that most of the B block sequences outside of the previously determined invariant G1, T3, C4 and R5 positions (11, 19, 24, 25, 26) can be altered with little effect on transcription. Our data indicate that changes in the 3' half of the consensus sequence affect promoter function less than changes in the 5' half. Transcription of mutants with changes 3' to position R5 was

approximately equivalent to that of the wildtype template. However, certain mutations 5' to position R5 resulted in large decreases in transcription. In particular the two G's at positions -1 and -2 are important functional components of the VA promoter. Mutants m232-44 and m232-65 change G(-2) to an A, resulting in a significant drop in transcription. Changing G(-1) to an A has no noticeable effect. However, the change from a purine to a pyrimidine (in m232-85 and m232-102) results in an almost total loss of transcription activity. Changes in nearby upstream nucleotides have less influence on transcription levels. Deleting position G(-3) (mutant 232-81), thus converting this position to a pyrimidine, has no effect on promoter strength.

B Block clustered mutants

The results of the analyses of clustered mutations in the B block are shown in Fig. 6B. Unlike the A block, the B block does not accommodate drastic changes in nucleotide sequence. At least one invariant site within the B block consensus sequence has been changed in each of the clustered mutants tested, and none of these displays more than 10% of wildtype transcription activity. Analysis of mutant 241-66 suggests the requirement for a purine at the G(-2) position. This mutant, which contains G to T changes at positions -1 and -2, shows almost no transcriptional activity, while m232-85, in which G(-1) is changed to a T, has 10% activity.

## DISCUSSION

In the experiments reported here, random point and clustered mutations in the Ad-2 VA I gene were generated and examined for their effect on the function of the VA I intragenic promoter. Previous studies of this promoter have demonstrated that most of the sequences outside of the B block are not required for efficient transcription. Neither mutation of non-B block sequences upstream of the BamHI site nor deletion of sequences downstream of this site has a significant effect on promoter function (5, 6, 20, 27). In our experiments, point mutations were restricted to the region of the gene 5' to the BamHI site, while clustered mutations were generated only in the A and B block regions. Promoter function in the mutant templates was tested by *in vitro* transcription assays. Our results support and add to previous findings which suggest that mutations in the 5', A block, and interblock regions of the VA I gene are not as detrimental to promoter function as B block mutations.

The deletion of 5' sequences to within 10 base pairs of the A block has been shown not to prevent transcription (6). The interblock sequence appears to have little or no effect on transcription when subjected to point mutations or even complete substitution by foreign spacer DNA (20). Comparisons between naturally occurring adenovirus type 2 and simian adenovirus type 7 VA genes show great variation in the interblock region but not within the blocks themselves (24). Results of varying the ~33bp spacer lengths have shown that it can be lengthened to ~60 bps or shortened by several base pairs without greatly disturbing promoter strength (20). It has been suggested that this size constraint is related to the binding and

interaction of transcription factors with the promoter sequence. In agreement with these results, we have found that point mutations in the 5' or in the interblock region do not have a significant effect on promoter function.

Our results indicate that even clustered mutations in the A block are not able to prevent transcription. This suggests that any single nucleotide of the A block may not be as important as the total sequence composition for transcription of the VA I gene. This is in contrast to some yeast tRNA genes, in which single point mutations in the A block result in a significant reduction in promoter strength (21, 22, 23). One explanation for our results would be that the A block functions at some secondary level of interaction during the formation of transcription complexes. This hypothesis is supported by a previous study (29) which used a series of deletion mutants to define the organization of the VA I transcriptional control region.

For tRNA and VA I genes the primary step on the path to transcription complex formation involves the interaction of transcription factor IIIC with B block sequences (6). Not surprisingly many of our mutations in the B block have dramatic effects on promoter activity. Our results indicate that VA I promoter function is most sensitive to mutations in nucleotides G(-1) to R5 of the B block. We have also noted that changing position G(-2) to an A causes a decrease in promoter strength. However, conversion of position C(-4) to a T has little effect on transcription.

It is remarkable that single point mutations in the invariant positions of the B block have a significant effect on promoter strength. This implies that transcription factors recognize either individual nucleotides or DNA secondary structures which are altered by small changes in the nucleotide sequence. It will be interesting to analyze the effects of mutations in the B block on local structure and to correlate these structural changes with changes in promoter function.

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