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Transcriptional Properties of BmX, a Moderately Repetitive Silkworm Gene That Is an RNA Polymerase III Template

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We analyzed the transcriptional properties of a repetitive sequence element, BmX, that belongs to a large gene family (~2 × 10^4 copies) in the genome of the *Bombyx mori* silkworm. We discovered BmX elements because of their ability to direct transcription by polymerase III in vitro and used them to test the generality of the properties of previously identified silkworm polymerase III control elements. We found that the signals that act in *cis* to control BmX transcription strongly resemble those that direct transcription of other silkworm polymerase III templates. As with silkworm tRNA and 5S RNA genes, transcription of BmX requires sequence signals located both upstream and downstream from the site of transcription initiation. The critical upstream sequences are structurally as well as functionally similar in the three kinds of templates. The downstream control region of BmX resembles the corresponding part of a silkworm alanine tRNA gene in that it provides a large (>100 base pairs) region that influences transcription factor binding. Moreover, the factor-binding regions of both $tRNA^{Ala}$ and BmX genes are remarkable in that under certain conditions, key elements within them (the B boxes, for example) appear dispensable. This behavior can be understood if, in both of these templates, the downstream control region acts as a large target for interaction with a multifactor complex.

In recent years, it has become apparent that the genomes of higher eucaryotes contain many families of repeated sequence elements (16, 24, 27, 32), at least some of which are functional as templates for transcription. Though in most cases the biological consequence of such transcription is not known, the abundance of the repeated sequences and their ubiquitous distribution in a variety of organisms argue for their functional importance. In several cases, transcription of repeated elements by polymerase III has been suggested to influence transcription of nearby genes by polymerase II (4, 7, 20). Thus, it is important to determine the functional characteristics of repeated sequences that are capable of serving as templates for transcription.

We have discovered and functionally analyzed a repetitive sequence element in the genome of the silkworm, *Bombyx mori*. We call this element BmX (for *Bombyx mori* element X). As we show in this report, the BmX element belongs to the class of repetitive elements called Bm1 that was isolated by Adams and his colleagues on the basis of its rapid renaturation kinetics (1). Its abundance (~2 × 10^4 copies in the 5 × 10^8-base-pair [bp] silkworm genome) accounts for its fortuitous presence on many of the large (5- to 10-kilobase [kb]) genomic DNA fragments that we had isolated in the course of other work. Although the BmX element does not belong to either of the best known classes of polymerase III templates (tRNA genes and 5S RNA genes), our preliminary work showed that it is capable of directing transcription by *Bombyx* RNA polymerase III in vitro. Transcription of BmX displays the α-amanitin resistance characteristic of isolated *Bombyx* RNA polymerase III (28). Moreover, since the work of Adams et al. (1) showed that Bm1-related RNA accumulates in vivo, it seemed likely that BmX elements are bona fide templates for polymerase III. We reasoned, therefore, that analysis of the transcriptional properties of this novel class of templates could help to delineate features of polymerase III control elements that are of general importance.

In particular, we wanted to know whether the signals that direct BmX transcription in *cis* exhibit the two most remarkable features of the signals that govern transcription of tRNA and 5S RNA genes in silkworms. One of these features is the absolute requirement for a short upstream sequence element for transcription of both tRNA and 5S RNA genes (8, 17, 22). A second striking feature of *Bombyx* polymerase III control elements—one that, so far, has been noted only in tRNA genes—is the contribution of an unexpectedly large downstream region (33). Not only is the downstream control element of a *Bombyx* $tRNA^{Ala}$ gene large (~125 bp), but it also possesses the unexpected property of appearing to shrink when measured under conditions that permit saturation of the transcription machinery by the template. The behavior of the downstream element is consistent with the idea that it provides a large binding region with which a complex of transcription factors interacts (23, 30). This view differs from a current one in which polymerase III transcription is driven by the interaction of individual transcription factors with small (~10- to 30-bp) elements located entirely within the coding region (reviewed in reference 14). It is important to determine whether the more extensive control elements we found in silkworm tRNA genes are characteristic of silkworm polymerase III templates generally.

MATERIALS AND METHODS

Cloned genes used in this work. The wild-type BmX gene used in this work was initially isolated on a 14-kb *Eco*RI fragment of the *Bombyx* genome. It was then subcloned as a 5.8-kb *Hin*dIII fragment in the plasmid pBR322 and subsequently as a 635-bp *Acc*I-*Sac*I fragment (made blunt ended through the action of bacteriophage T4 DNA polymerase [19]) inserted in both orientations at the *Sma*I site of the bacteriophage M13mp9. The structures of these two subcloned fragments are shown in Fig. 1a. The nucleotide sequence of both strands of the 635-bp *Acc*I-*Sac*I fragment shown in Fig. 1b was determined by analyzing appropriate

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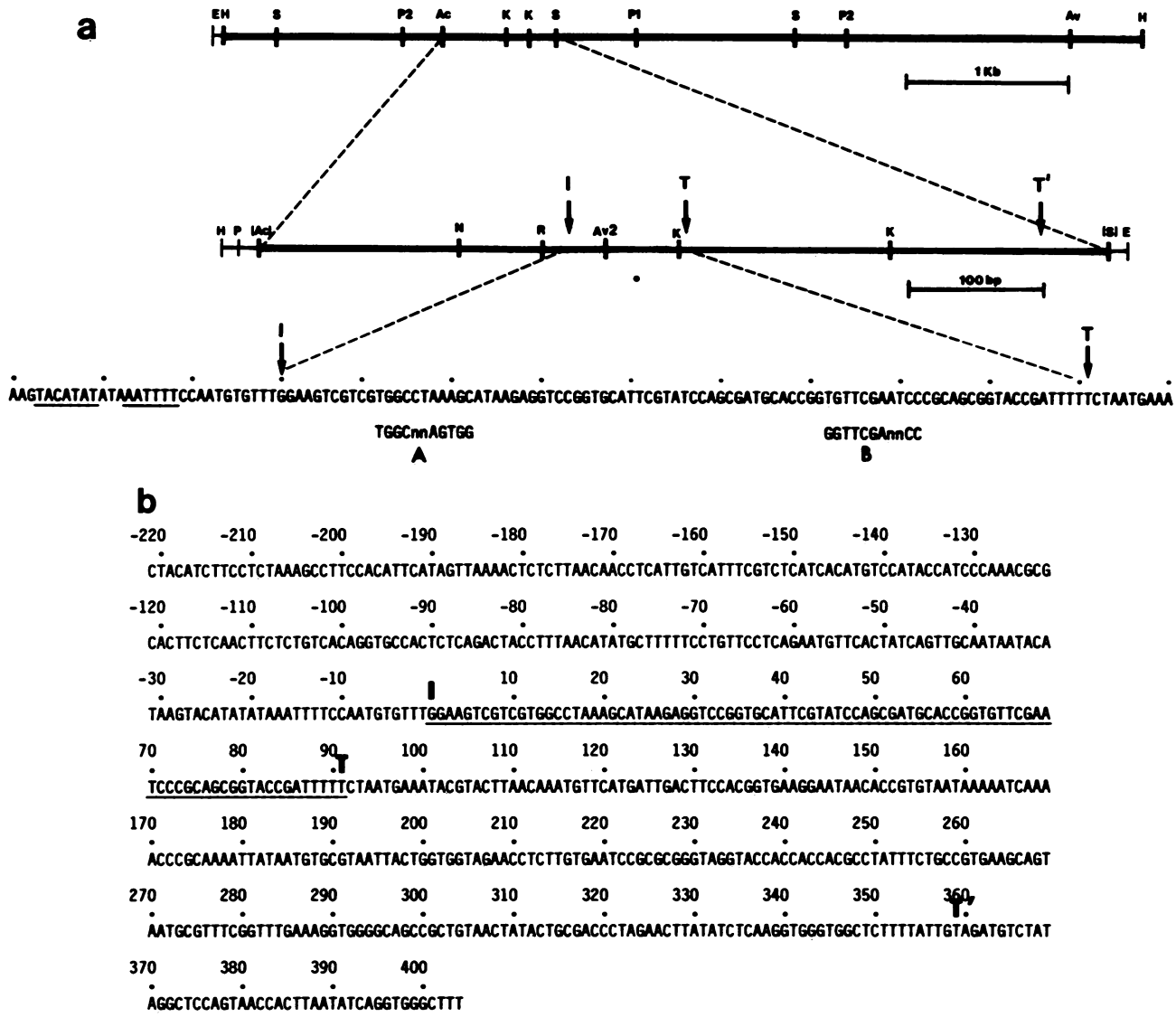

FIG. 1. (a) Structure of the BmX gene. The top two lines are restriction maps of a 5.8-kb *Hin*dIII fragment and a 635-bp *Acc*I-*Sac*I fragment of *Bombyx* DNA containing a BmX sequence. *Bombyx* DNA and vector DNA are indicated by thick and thin horizontal lines, respectively. The orientation of the *Bombyx* DNA fragments relative to vector sequences is indicated by the position of restriction sites in the vector DNA. The R orientation of the *Acc*I-*Sac*I fragment is shown. Restriction sites that were destroyed by the treatment used to create blunt ends are shown in parentheses. Restriction endonucleases used were Ac, *Acc*I; Av, *Ava*I; Av2, *Ava*II; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; N, *Nde*I; P, *Pst*I; P1, *Pvu*I; P2, *Pvu*II; R, *Rsa*I; S, *Sac*I. At the bottom of the figure is the nucleotide sequence of part of the BmX gene (−30 to +100). The noncoding strand is shown. The sites where transcription of the BmX gene is initiated (I) and terminated (T, T′) are indicated. Consensus A and B box sequences (11) are aligned below the BmX sequence. Oligonucleotides common to the sequences upstream of other *Bombyx* RNA polymerase III templates are underlined. (b) Complete sequence of the 635-bp *Acc*I-*Sac*I fragment that contains BmX. The noncoding strand is shown, and nucleotides are numbered from the site of transcription initiation (+1). The symbols I, T, and T′ are as described in panel a, and the sequence corresponding to the short (91-nucleotide) BmX transcript is underlined.

subcloned fragments by the dideoxy nucleotide method (26). The 635-bp subclone retains 231 bp of 5′-flanking DNA and 313 bp of 3′-flanking DNA. The transcriptional activities of BmX genes on both subcloned fragments were identical to that of the BmX gene on the original genomic fragment. Therefore, these subclones were used as wild-type standards for this work. The location of the BmX gene within cloned fragments of the *Bombyx* genome was established by hybridization of size-fractionated DNA fragments to labeled BmX RNA produced by transcription in vitro (29, 31).

Sequence analysis of BmX transcripts. Nucleotide sequence analysis of internally labeled RNAs was done by the fingerprinting methods developed by Sanger and his colleagues. (26). Oligonucleotides produced by digestion with T_1 RNase were fractionated two dimensionally, using homochromatography on polyethyleneimine thin-layer plates in the second dimension. The sequence of each T_1 oligonucleotide was determined by analysis of the products of secondary and tertiary digestion with RNases A and T_2. The 5′-terminal nucleotides of BmX RNAs were identified after intact transcripts had been digested to mononucleotides (21) and the resulting products separated by chromatography on polyethyleneimine thin-layer plates in 1 M KH_2PO_4, pH 3.5. The identity of the 5′-terminal nucleotide was confirmed by

treating the isolated nucleoside tetraphosphate with P1 nuclease to yield pppX and P_i. Analysis of transcripts that had been labeled with each of the four [α-^{32}P]nucleoside triphosphates in turn also allowed identification of the penultimate 5'-terminal nucleotide, as a consequence of the transfer of the α phosphate on the nearest neighboring nucleotide to the P_i released from the terminal nucleotide by P1 nuclease.

Construction of partially deleted derivatives of the BmX gene. A series of partially deleted derivatives of the BmX gene was constructed by BAL 31 exonuclease digestion (18). Mutants deleted from the 5' direction were produced from two different starting materials. One was the 635-bp *Acc*I-*Sac*I fragment inserted in M13mp9 in the orientation shown in Fig. 1. This fragment extends from −231 to +404 (numbered relative to the transcription initiation site) and in this orientation is designated *AccSac* WT-R. The other starting material used to produce 5' deletion mutants was a 430-bp *Rsa*I-*Sac*I fragment extending from −26 to +404 that had been inserted into the *Sma*I site of a derivative of M13mp9 that contained *Eco*RI sites on both sides of the *Sma*I site. Mutants deleted from the 3' direction were produced from the *Acc*I-*Sac*I fragment inserted in the opposite orientation at the *Sma*I site of M13mp9 (*AccSac* WT-L). To generate both sets of deletions of the *Acc*I-*Sac*I fragment, replicative-form DNA was digested first with *Hin*dIII, then with BAL 31 exonuclease, and finally with *Eco*RI and cloned into M13mp18 that had been cut with *Hin*cII and *Eco*RI. To generate deletions of the *Rsa*I-*Sac*I fragment, replicative-form DNA was digested with *Eco*RI and then with BAL 31 and cloned into M13mp9 that had been cut with *Sma*I. The positions of all deletion endpoints were established by DNA sequence analysis (26). Mutations generated from the *Rsa*I-*Sac*I fragment were deleted at both ends of the BmX insert. Sequence analysis showed, however, that in no case were more than 11 of the most distal bases (+393 to +404) removed. In the 3' deletion series, care was taken to ensure that an effective transcription termination site was provided in the vector sequences that replaced silkworm DNA. On templates in which deletion had removed the normal termination site, transcription stopped instead at the cluster of four T residues at positions 26 to 29 in the *lacZ* coding region of M13mp18.

Several different DNA sequences were used to replace *Bombyx* DNA at each of the following positions. (i) −3. One −3 deletion was generated, as described above, by BAL 31 digestion of *Bombyx* DNA from position −26. In this case, sequences extending from the *Sma*I site toward the *Eco*RI site in M13mp19 were joined to the remaining BmX sequence at −3, and the resulting clone was called −3mp19. Another substitute sequence that differed from this one at positions upstream of −21 was produced by transferring the *Eco*RI-*Hin*dIII segment containing this mutant BmX gene to M13mp18 to yield clone −3mp18. A different −3 deletion was created by using the 3'→5' exonuclease activity of bacteriophage T4 DNA polymerase (19) to remove BmX DNA between −26 and −3. The starting material for this mutagenesis was a 107-bp *Rsa*I fragment extending from −26 to +81 cloned into the *Sma*I site of M13mp9 such that the 5' end of the BmX gene was adjacent to the *Eco*RI site in the vector. Replicative-form DNA was digested with *Eco*RI and then incubated with T4 DNA polymerase in the presence of dCTP as the only deoxynucleoside triphosphate. After treatment with mung bean nuclease (P-L Biochemicals, Inc., Milwaukee, Wis.) to remove single-stranded ends, the DNA was recircularized by ligation. Deletion of BmX sequences extended farther than expected (to −3 instead of −6), presumably because of irregularities in the reactions catalyzed by DNA polymerase or by mung bean nuclease. Normal *Bombyx* sequences downstream of +81 were restored by standard recombinant DNA techniques that took advantage of a unique *Ava*II site at +31 in the BmX-coding region. The resulting clone was called −3mp9.

(ii) +57. The sequence replacing *Bombyx* DNA immediately downstream from +57 was altered by excising the original vector sequence with restriction enzymes and then recircularizing the DNA. Specifically, DNA containing the 3' deletion mutation extending to +57 was digested with a combination of *Pst*I and *Sph*I. The resulting protruding 3' ends were removed by the 3'→5' exonuclease activity of T4 DNA polymerase in the presence of all four deoxynucleoside triphosphates (19). Mutant tRNA genes containing this altered replacement sequence are designated +57-Alt.

(iii) +84 and +86. A natural *Kpn*I site was used to insert three different sequences in place of the normal one downstream of +84 or +86. First, *AccSac* WT-R DNA was cut with *Hin*dIII and *Kpn*I, and the ~340-bp fragment containing the 5'-flanking region and the coding region of BmX to +84 was inserted into M13mp19 that had been cut with *Hin*dIII and *Kpn*I. This clone is called +86-Rep (for replacement), and it is deleted for the BmX sequence to +86 rather than +84 because of fortuitous reconstruction of part of the normal sequence by vector DNA. Two other sequences were juxtaposed at +84 by cutting *AccSac* WT-R DNA with *Kpn*I alone and then recircularizing the molecules with T4 DNA ligase. Two different products were obtained. In one (+84-0), the *Kpn*I fragment extending from +84 to +238 was lost, and in the other (+84-Inv), it was inverted relative to its normal orientation.

Transcriptional activity measurements. The transcriptional activity of wild-type and mutant BmX genes was measured in vitro with extracts of *Bombyx* silkgland nuclei in the presence of nonspecific vector DNA and subsaturating concentrations of template as previously described (33). Typical transcription rates from wild-type BmX genes were 50 transcripts per gene per h. The transcriptional activity of mutant genes was normalized when appropriate for differences in transcript length.

Determination of competitive ability. The ability of a mutant gene to reduce transcription from a reference gene was measured relative to the ability of a wild-type gene to reduce transcription from the same reference gene in the same experiment. This relative value is expressed as a percentage of wild-type competitive ability (33). In all cases, the competitor and the reference gene were added to the reaction mixtures simultaneously. Two different reference genes (+84-0 and +84-Inv) encoding BmX primary transcripts whose lengths differ from wild type were used in these experiments. Both of these mutant genes retain wild-type competition strength. No differential influence of the two reference genes on competition strength measurements was observed. Competitor DNAs were tested at both five and eight times the molar concentration of the reference gene.

RESULTS

Identification of BmX transcription unit. We analyzed both structural and functional properties of one copy of the element we call BmX. Since the existence of BmX was originally signalled by the synthesis of its transcripts in vitro, our structural analysis relied initially on using BmX transcripts as hybridization probes to localize the DNA segment that encoded them. The particular BmX element we ana-

lyzed directs the synthesis of two small RNAs. The location of these transcripts within a 5.8-kb *Hin*dIII fragment of the *Bombyx* genome is shown in Fig. 1. This analysis first mapped both BmX RNAs to a 635-bp *Acc*I-*Sac*I fragment and then mapped the shorter RNA to a region of about 100 bp (an *Rsa*I-*Kpn*I fragment) within the larger fragment. The nucleotide sequences of both strands of the 635-bp *Acc*I-*Sac*I fragment were determined.

To find the exact position of the BmX element within this sequence and to determine the relationship of the two BmX RNAs to each other, we examined the structure of both BmX transcripts by fingerprinting methods (3). In parallel experiments, these transcripts were labeled internally with each of the four ribonucleotides, separated electrophoretically, and then digested with RNase T_1. The resulting oligonucleotides were fractionated to produce a characteristic fingerprint, and the sequence of each oligonucleotide was determined by secondary and tertiary digestion. This analysis permitted unambiguous alignment of the BmX oligoribonucleotides with the DNA sequence. It established that the 5′ ends of both BmX RNAs map to the same small region (I in Fig. 1) but that the two transcripts extend different distances downstream (to T at +91 or T′ at +354 in Fig. 1). This sequence analysis also showed that the BmX element we studied is a member of the Bm1 gene family reported by Adams et al. (1). The Bm1 consensus sequence 2 begins at position +2 in BmX and includes the entire transcribed region of BmX (+1 to +354) plus sequences farther downstream. Within the region where data are available for both BmX and Bm1 (+2 to +404 in BmX), 93% of the nucleotides are identical.

To learn whether the BmX RNAs are primary transcripts, and if so, to determine exactly where transcription initiates, we examined the 5′ termini of the BmX RNAs in detail. Digestion of both BmX RNAs to mononucleotides yielded triphosphorylated 5′ ends characteristic of primary transcripts (Fig. 2). The 5′-terminal nucleoside tetraphosphates produced from both transcripts are labeled only by [α-^{32}P]GTP, despite the fact that they contain α phosphates derived from both the terminal and the penultimate nucleotide. This result establishes that the 5′-terminal sequence of BmX RNAs must be pppGpGp. Since there is only one GpG sequence whose position in BmX-containing DNA is consistent with the length and the T_1 oligonucleotide composition of the BmX RNAs (see sequence in Fig. 1), we conclude that synthesis of both of these transcripts initiates precisely at the nucleotide marked I in Fig. 1. Transcription initiation at other sites was not detected, and we estimate that alternate initiation sites would have been detectable had they been used at least 10% as frequently as this one.

The structural analysis indicated that the two BmX transcripts have a common 5′ end but differ at their 3′ ends. In principle, different 3′ ends could be generated either by multiple transcription termination events or by posttranscriptional processing. We favor the idea that variable transcription termination is responsible and that the longer BmX RNA is produced when the transcription apparatus proceeds through the first termination site (T) and stops downstream at T′ instead. Two pieces of evidence support this interpretation. (i) The presence of a U-rich 3′ terminus typical of a polymerase III primary transcription product (6) is consistent with creation of the short BmX transcript by a transcription termination event rather than by a processing event. (ii) The relative proportions of the two BmX transcripts are determined by the concentration of UTP present during the transcription reaction. High concentrations of UTP (~600 μM) favor synthesis of the long transcript, whereas low concentrations (25 μM) favor synthesis of the short one (data not shown). This phenomenon has been observed previously (13) and has been interpreted to indicate that low concentrations of UTP increase the probability that termination will occur at the first site where multiple U residues must be incorporated into the transcript.

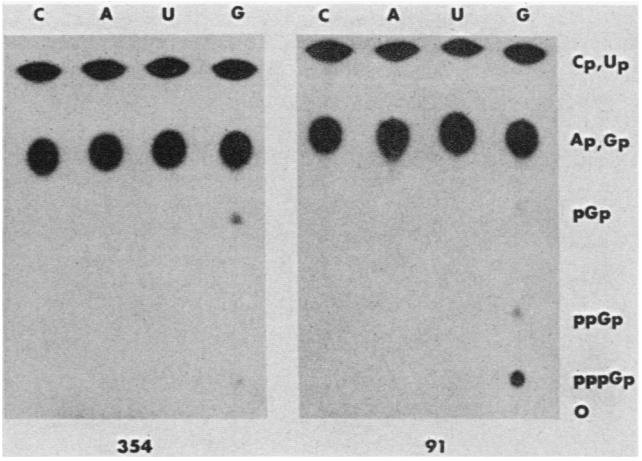

FIG. 2. Identification of the site where BmX transcription initiates. Short (91-nucleotide) or long (354-nucleotide) BmX transcripts were labeled separately with each of the four nucleotides (C, A, U, G, as indicated at the top of the figure) and digested to mononucleotides, and the resulting products were chromatographed on polyethyleneimine thin-layer plates. The positions of the mononucleotide products relative to the origin (O) are shown. Only those transcripts labeled with G yielded a digestion product with the slow mobility expected of a 5′-terminal nucleoside tetraphosphate (pppNp). The identities of the products shown here were determined by comparison of their chromatographic properties with marker nucleotides and by subsequent digestion with P1 nuclease (see Materials and Methods). In particular, the structure of the presumptive 5′-terminal nucleoside tetraphosphate (pppGp) produced from G-labeled BmX transcripts was conclusively identified by using P1 nuclease to remove the 3′ phosphate from pppNp. The major labeled products of P1 digestion were pppG and P_i, as determined by comigration with markers on polyethyleneimine plates.

Transcriptional properties of BmX. To identify the sequences required for transcription of BmX in vitro, we constructed a series of partially deleted derivatives of BmX in which successively larger amounts of the gene were removed from either the 5′ or the 3′ direction. We measured both the transcriptional activity of the resulting mutant BmX templates and their ability to compete for essential transcription factors. Figure 3 shows the results of this analysis. Figure 3a shows the transcripts produced by wild-type BmX genes and by representative mutant derivatives. In the cases in which multiple transcripts were synthesized, all of them were included in the measurement of the molar amount of transcript produced. Removal of normal sequences from −231 to −36 did not reduce transcriptional activity (Fig. 3b, open symbols). Removal of wild-type sequences between −36 and −15, however, drastically reduced transcriptional activity. Mutant genes with 15 or fewer base pairs of normal 5′-flanking DNA were completely inactive. To determine whether downstream sequences contribute to BmX transcription, we tested 3′ deletion mutants of the BmX gene for transcriptional activity. Although loss of wild-type sequences from +404 to +238 did not affect activity, loss of sequences from +238 to +72 caused a moderate but reproducible reduction (from 100 to ~75% of the wild-type level)

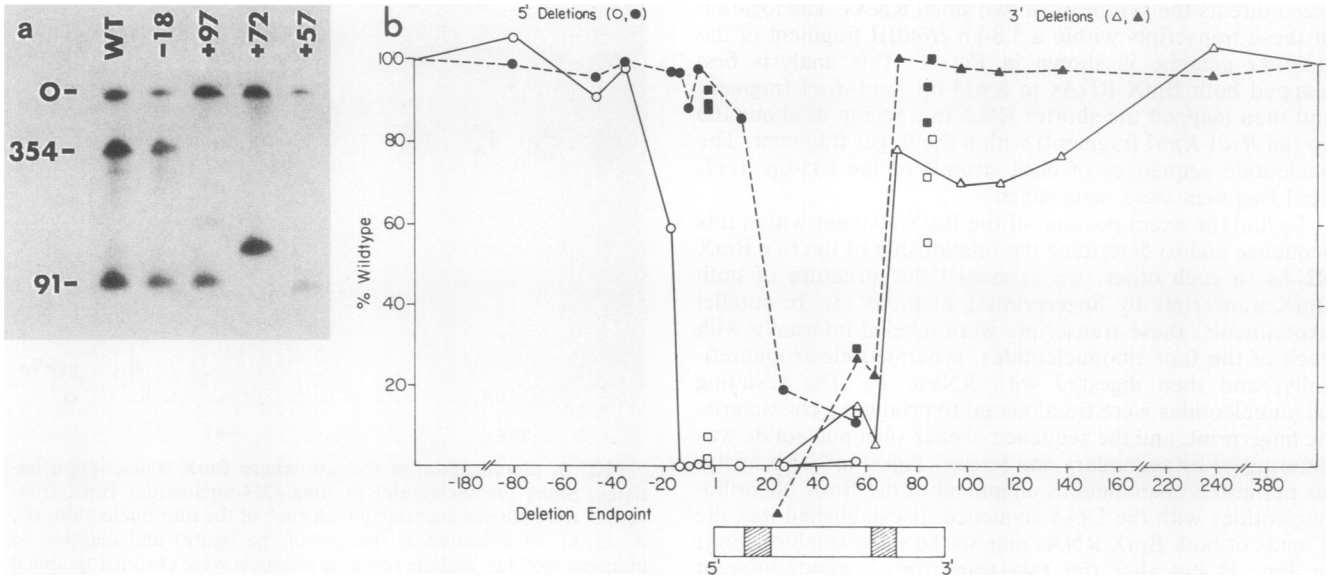

FIG. 3. (a) Transcripts produced in vitro from wild-type (WT) and mutant BmX genes. Transcription was done at subsaturating concentrations of template as described in Materials and Methods. The transcripts shown were resolved by electrophoresis in 8% polyacrylamide gels (22). The gel origin is marked (O), as are the positions of the two major transcripts (91 and 354 nucleotides long, as determined by RNA sequence analysis) produced by wild-type BmX. The transcription products from wild-type BmX (WT), one 5′ deletion mutant (−18), and three 3′ deletion mutants (+97, +72, +57) are shown. (b) Identification of BmX gene sequences that contribute to transcriptional activity and to competitive strength. The transcriptional activity of partially deleted genes (as a percentage of the activity of wild-type genes) is represented by open symbols (5′ deletions, ○, □; 3′ deletions, △, □), and the competitive ability of the same genes (as a percentage of the wild-type value) is represented by closed symbols (5′ deletions, ●, ■; 3′ deletions, ▲, ■) at the position of the deletion endpoint on the horizontal axis. Deletion endpoints are numbered with respect to the transcription initiation site. Negative values indicate distance upstream; positive values indicate distance downstream. For reference, the BmX primary transcript is shown diagrammatically below the horizontal axis. The extent of A and B boxes in other genes transcribed by RNA polymerase III is shown by hatching. The activities and competition strengths of mutants in which the *Bombyx* sequence is replaced by sequences other than the most commonly used one in M13mp18 (see Materials and Methods) are indicated by squares. Experiments were done in the presence of a constant amount of total DNA (0.25 μg). Transcriptional activities were measured at a template concentration of 5.5×10^{-11} M. Competition experiments were as described in Materials and Methods. The number of determinations on which each of the mean transcriptional activity and competitive ability values is based (*n*) and the standard deviation of each mean (*s*) are given below in parentheses after the deletion endpoints (*n,s*). To allow discrimination among the results for mutant genes containing different replacement sequences, the numerical activity value plotted on the graph is also given below in parentheses immediately after the name of each of these mutants. Transcriptional activity of 5′ deletions: −81 (9, 13); −48 (6, 7); −36 (12, 13); −18 (11, 5); −15 (6, 2); −11 (6, 2); −7 (6, 3); −3mp9 (= 2%) (6, 2); −3mp19 (= 1%) (6, 1); −3mp18 (= 7%) (8, 2); +10 (6, 1); +27 (6, 3); +56 (6, 1). Transcriptional activity of 3′ deletions: +238 (10, 14); +137 (12, 9); +112 (5, 6); +97 (9, 8); +86-Rep (= 81%) (6, 7); +84-Inv (= 72%) (10, 11); +84-0 (= 55%) (6, 5); +72 (12, 11); +63 (9, 2); +57 (9, 7); +57-Alt (6, 1); +25 (6, 1). Competitive ability of 5′ deletions: −81 (6, 3); −48 (6, 3); −36 (6, 3); −18 (12, 3); −15 (12, 7); −11 (6, 3); −7 (12, 7); −3mp9 (= 93%) (6, 1); −3mp19 (= 88%) (6, 3); −3mp18 (= 89%) (6, 1); +10 (6, 1); +27 (12, 11); +56 (6, 4). Competitive ability of 3′ deletions: +238 (6, 3); +137 (12, 8); +112 (6, 1); +97 (6, 2); +86-Rep (= 101%) (6, 2); +84-Inv (= 85%) (6, 4); +84-0 (= 94%) (6, 3); +72 (6, 2); +63 (6, 8); +57 (9, 9); +57-Alt (6, 3); +25 (6, 7).

and loss of sequences from +72 to +63 resulted in a precipitous drop in activity (to 5% of the wild-type level).

We ruled out the possibility that the observed reduction in transcriptional activity is only apparent and is actually due to instability of the RNA products in cases in which the mutation alters the structure of the transcript. Altered transcripts are produced by members of the 3′ deletion series in which the normal transcription terminator is replaced by a different sequence. The RNA products of such mutant templates were tested for stability by reincubation of isolated transcripts under typical transcription conditions. No differential instability of wild-type as compared with mutant RNA molecules was detected (data not shown). We have also demonstrated, by the type of analysis we applied to transcripts from wild-type BmX genes, that the transcripts from mutant genes (5′ deletion −18 and 3′ deletions +57 and +72) initiate at the normal position (data not shown).

The reduction in transcriptional activity exhibited by any of the mutants could be due either to the removal of a positively acting element or to the introduction of a negatively acting one. It is probable that a positive effector has been removed since substitution by several different replacement sequences at each of three locations (−3, +57, and +84 or +86) did not change the transcriptional activities observed (Fig. 3b). It is unlikely that all the sequences used to replace the normal sequence would fortuitously create effective negative elements.

Competition between mutant and wild-type genes provides a simple way to identify sequences that contribute to transcription factor binding. What is measured is the ability of one gene (the competitor) to sequester factor(s) from another gene (the reference) when both genes are added to a reaction mixture simultaneously. The competitive ability of mutant genes is compared with the competitive ability of a wild-type gene measured in the same experiment. Analysis of the competitive ability of partially deleted *Bombyx* tRNA genes showed that 3′-coding and 3′-flanking sequences are important for binding transcription factors (33). In that work, we demonstrated that the competition we observed was for a DNA-binding transcription factor. Competition between

BmX and tRNA genes established that BmX genes compete with $tRNA^{Ala}$ genes for the same limiting DNA-binding component (data not shown). To discover which BmX sequences are important for binding this factor, we measured the competitive ability of deletion mutants of the BmX gene.

In Fig. 3b (closed symbols), the competitive ability of partially deleted genes is plotted with respect to the deletion endpoint. These data show that the region that contributes to transcription factor binding lies within the BmX gene, extending from ~+10 to +72. Comparison of the curves describing transcriptional activity and competitive strength shows that the sequences that contribute to transcription factor binding occupy only part of the full region that contributes to transcriptional activity. 5'-flanking sequences that are important for the transcriptional activity of the BmX gene do not contribute to factor binding. Specifically, removal of sequences from the 5' side did not reduce binding ability until all 5'-flanking DNA and ~10 bp of coding sequence was removed as well. As sequences between +10 and +27 were removed, binding strength dropped sharply (from 90 to 20% of the wild-type level). Similarly, removal of 3'-flanking sequences that contribute to transcriptional activity (+238 to +72) did not reduce transcription factor-binding strength. As coding sequences upstream of +72 were removed, however, binding strength was lost rapidly. Deletion of sequences between +72 and +63 reduced competitive ability to 23% of the wild-type level.

Our previous analysis of $tRNA^{Ala}$ genes (33) revealed a curious feature of the downstream control region. Specifically, the apparent extent of this region depended on the concentration of template used to determine the transcriptional phenotype of partially deleted tRNA genes. As long as concentrations of template that did not saturate the transcription apparatus were used, the same large region was observed. When concentrations of template high enough to saturate the transcription apparatus were used, however, the full extent of the control region was obscured. When the template concentration exceeded saturation by greater and greater increments, successively smaller regions of the $tRNA^{Ala}$ gene appeared to be sufficient to direct transcription. Figure 4 shows that the BmX gene exhibits the same kind of behavior. The size of the BmX control region varied dramatically as a function of template concentration. At subsaturating concentrations of template, the BmX control region appeared to extend from ~−18 to at least +137, whereas at saturating template concentrations it had nearly the same upstream boundary, but extended only to ~+63.

DISCUSSION

The structure of the BmX element suggests that it has the potential to serve as a template for RNA polymerase III. Our work shows that BmX in fact directs transcription in vitro and that it does so in a fashion that is typical of the more classical polymerase III templates derived from silkworms. In particular, transcriptional analysis of partially deleted derivatives of the BmX gene showed that the region that contributes to BmX transcriptional activity is quite large. It extends from an upstream boundary located between −36 and −18 to a downstream boundary between +137 and +240. Thus, the minimum size of the BmX control region is 157 bp.

The size of the BmX control region resembles that of the *Bombyx* $tRNA^{Ala}$ gene (~160 bp) we analyzed recently (33). Sequences required for maximal transcription of the $tRNA^{Ala}$ gene extend from at least −13 to +146. In addition to being similar in overall size, the BmX and $tRNA^{Ala}$ control elements are alike in containing functionally distinguishable subregions. That is, in both genes the role of 5'-flanking regions is different from the role of elements located farther downstream. Measurements of the ability of partially deleted genes to compete for the limiting transcription factor(s) in crude extracts indicate that the downstream control elements contribute to transcription factor binding. The upstream elements either do not contribute to factor binding at all or do so for transcription factors that are present in excess. In either event, the important point is that both BmX and $tRNA^{Ala}$ genes exhibit functional specialization within their control regions. It seems likely that whatever the function of the upstream element in BmX genes is, it is the same in a variety of *Bombyx* polymerase III templates. Sequences within the upstream region in a BmX gene (underlined in Fig. 1a) are very similar to those in the

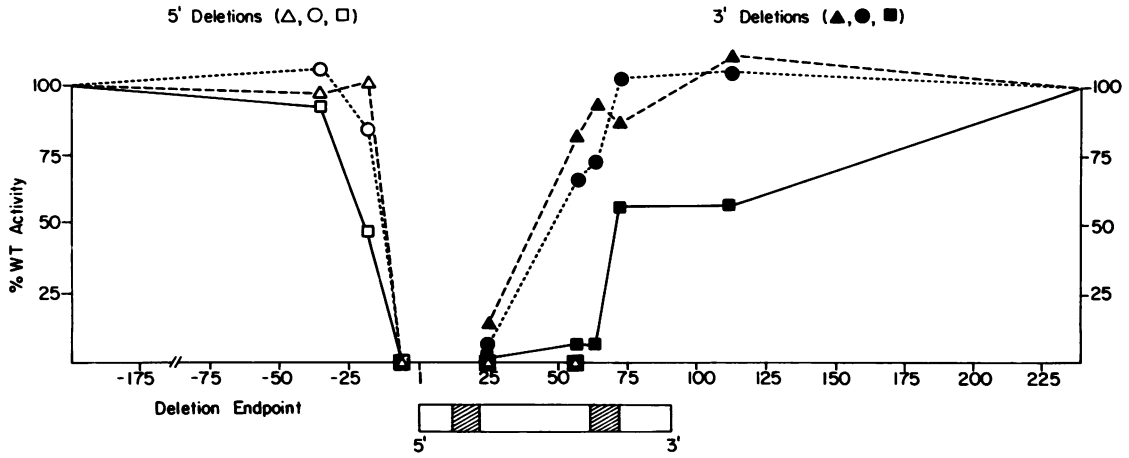

FIG. 4. Effect of template concentration on the transcriptional activity of partially deleted BmX genes. The transcriptional activity of partially deleted genes is represented by open symbols (5' deletions) or closed symbols (3' deletions) at the positions of the deletion endpoints on the horizontal axis. The diagram of the BmX primary transcript below this axis is explained in the legend to Fig. 3b. Experiments were done in the presence of a constant amount of total DNA (0.25 μg) and at three template concentrations: 1.75×10^{-9} M (△, ▲), 8.75×10^{-10} M (○, ●), 5.5×10^{-11} M (□, ■).

corresponding positions of a 5S RNA gene (22) and three tRNA genes (8, 17). These results strongly suggest that the upstream control element provides a signal that is generally required for transcription by *Bombyx* polymerase III.

To a first approximation, the downstream control elements of BmX and $tRNA^{Ala}$ genes are similar. Competition experiments show that these regions contribute to transcription factor binding in both genes. It is probable, however, that the details of factor-gene interactions differ in the two templates. This idea is suggested by differences at the downstream boundaries of their control elements. For the $tRNA^{Ala}$ gene, sequential removal of all downstream sequences resulted in a loss of transcriptional activity that was gradual and uniform (33). In the case of BmX, removal of 3′-flanking and distal coding sequences caused a gradual loss of activity, but removal of sequences between +72 and +63 produced a dramatic drop in activity (from 80 to 5% of the wild-type level). The region between +72 and +63 is nearly identical (8 of 9 bp) to a sequence known as the B box that has been shown to contribute to the transcriptional activity of various polymerase III templates in several organisms (2, 5, 9–12, 15, 25). The results of the BmX analysis suggest that for silkworm genes, the B box is more important to the transcription of BmX genes than it is to transcription of $tRNA^{Ala}$ genes.

Inspection of the 3′ boundary of the control region revealed an additional difference between BmX and $tRNA^{Ala}$ genes. In the $tRNA^{Ala}$ gene, the downstream boundaries of sequences important for transcriptional activity and for competitive strength coincide, whereas in the BmX gene they do not. Specifically, removal of BmX sequences between +137 and +72 reduced transcriptional activity but did not affect competitive ability. In contrast, similarly deleted $tRNA^{Ala}$ genes tested in the same extracts were equally impaired in these two functions. Conceivably, the transcription component that acts in the 3′-flanking region of BmX genes is distinct from the components that act on tRNA genes. Alternatively, the same components may act on both genes, but the relative concentrations required for them to do so may differ for the two templates. Thus, the concentration of the component that acts distally could be limiting for tRNA genes, but not for BmX genes.

Support for the idea that tRNA genes and BmX genes differ in their interactions with the transcription apparatus is provided by the observation that the two genes differ in their ability to direct transcription in heterologous systems. We have found that, unlike $tRNA^{Ala}$ genes, BmX templates are completely inactive either in extracts derived from *Xenopus* frogs or in intact *Xenopus* occytes (G. M. Ramahi, M.S. thesis, University of Oregon, Eugene, 1986; E. Wilson, unpublished data). Since transcription systems derived from *Xenopus* oocytes are able to transcribe genes encoding 5S RNA, tRNA, and other small RNAs from a variety of organisms, their inability to act on BmX genes may be significant.

Although the downstream parts of the control regions of BmX and $tRNA^{Ala}$ genes differ in detail, they share an interesting property. Both control regions display apparent shrinkage when measured under conditions of saturating template. This effect is particularly remarkable for the BmX gene in which, at subsaturating concentrations of template, the sequence between +63 and +72 (the B box) appears crucial both for transcriptional activity and for binding transcription factors. Deletions that extend through this interval from the 3′ side lower transcriptional activity to ~5% and competition strength to 20% of the wild-type levels. Nonetheless, at saturating concentrations of template, genes lacking this region direct transcription at 90% of the wild-type rate. That is, in contrast to what happens at low template concentration, under conditions of saturating template, the B box is dispensable for BmX transcription. Our interpretation of this paradox is that mutant genes lacking part of the factor-binding region retain sufficient contacts in the remainder of the control region to bind all the transcription components necessary for activity. We imagine that protein-protein contacts among these components can partially compensate for the loss of normal DNA-protein contacts. At low concentrations of template, we expect that the full transcription apparatus binds less efficiently to mutant genes than to wild-type genes. When the template concentration is high enough to saturate the transcription apparatus, however, binding to mutant genes may be driven to levels that approach the binding to wild-type genes. The fact that both tRNA genes and BmX genes exhibit this behavior suggests that it reveals a basic feature of the interaction between the template and the polymerase III transcription apparatus. It is consistent with the idea that a large part of the control region of both of these genes acts as a unit, providing a large target for interaction with a complex of transcription factors.

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