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Potentiation of a polyadenylylation site by a downstream protein–DNA interaction

(*Drosophila melanogaster*/gypsy/retroposon/suppressor of Hairy-wing/transposable element)

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ABSTRACT **The gypsy retroposon of *Drosophila melanogaster* contains a sequence that potentiates upstream polyadenylylation sites. In contrast to other sequences that influence poly(A) site use, it appears to operate at the level of the DNA template. Nuclear extracts contained protein that bound to a repeated motif in the DNA. Flies with mutations that reduced transcripts polyadenylylated in the 5′ long terminal repeat of gypsy contained less DNA-binding activity than wild type. A change in the repeat motif reduced both protein binding and poly(A) site potentiation. These findings provide evidence that DNA-binding proteins can regulate polyadenylylation sites.**

The 3′ ends of most eukaryotic mRNAs are formed by cleavage and addition of a poly(adenylic acid) tail. This affords opportunities for regulation of gene expression (1). To understand poly(A) site function, the sequence signals must be identified. The AAUAAA sequence occurring 10–30 nucleotides (nt) upstream of most higher eukaryote poly(A) sites (2) is essential (3–5) and sequences within 100 nt downstream are also important (3, 6–13). Downstream elements are often T+G-rich (3, 14) and their effects can be position dependent (15).

An example of regulated transcript truncation was found in the gypsy retroposon of *Drosophila melanogaster*. Many mutations in *Drosophila* are gypsy insertions (16). When gypsy is situated in the transcribed region of a gene, and oriented in the same transcriptional direction, most gene transcripts are polyadenylylated in the 5′ long terminal repeat (LTR) (17, 18). The phenotypes associated with most gypsy insertion alleles are less severe in the presence of mutations of the suppressor of Hairy-wing [*su(Hw)*] gene (16, 19). Mutation of *su(Hw)* reduces the level of 5′ LTR truncated transcript in both naturally occurring (17) and constructed gypsy insertion alleles (18).

By insertion of gypsy fragments into the intron of the *hsp82* heat shock gene, the sequences responsible for high levels of truncated transcript were found to be within a portion of the LTR and a non-LTR segment (18). The former contains a poly(A) site and the latter contains several repeats of the sequence YRYTGCATAYYY (Y, pyrimidine; R, purine). Several phenotypic revertants of gypsy insertion alleles have alterations in the repeat region, indicating that it is involved in the effects of gypsy on gene expression (20, 21). The repeat-containing segment differs from other downstream elements that influence poly(A) sites. It is further downstream (350–680 nt) and works in either orientation. Evidence presented here indicates that it operates through binding of protein to the repeat motif in DNA.

MATERIALS AND METHODS

Nuclear Extracts. Schneider 2 (S2) cell nuclear extract was made as described (22) except the dialysis buffer contained 100 mM NaCl. For DNase I footprinting, binding activity was partially purified. Extract was applied to a heparin-agarose column (1.0 × 7.7 cm) in buffer (CB; ref. 22) containing 0.1 M NaCl. The column was developed with 200 ml of CB containing a linear 0.1–1.5 M NaCl gradient. Fractions containing activity binding to the *Bal* I/*Bst*XI fragment of bx^{34e} gypsy (BaBx; ref. 18) were pooled, dialyzed against CB containing 20% (vol/vol) glycerol and 0.1 M NaCl, and concentrated by vacuum dialysis.

Pupal nuclear extracts were prepared from cells liberated from pupal cases by gentle mashing in cell culture medium. Pupal cells were broken open by mixing in a Vortex with acid-washed glass beads (1 mm) for 1.5 min at 4°C in hypotonic buffer (buffer I; ref. 22) and proteins were extracted from nuclei as described.

DNA–Protein Binding Analysis. The BaBx (*Bal* I/*Bst*XI) fragment of bx^{34e} gypsy was cloned into the *Sal* I site of pGEM-1 (Promega Biotec) with linkers. *Sal* I fragments were 5′-end-labeled with T4 polynucleotide kinase and [γ-^{32}P]-ATP, or 3′-end-labeled with Klenow polymerase and [α-^{32}P]deoxyribonucleotides, and purified by gel electrophoresis (23).

Binding reaction mixtures (25 μl) contained the indicated DNA fragments and extract and were incubated at 20°C–25°C for 15 min. All contained 10 mM Hepes (pH 7.9), 0.15 M NaCl, 1.5 mM $MgCl_2$, 0.5 mM phenylmethylsulfonyl fluoride, poly[d(IC)] (80 μg/ml), and 5% (vol/vol) glycerol. Except for most reactions in Fig. 1*A*, all contained pGEM-1 plasmid DNA (20 μg/ml) as competitor.

For gel mobility shift assays, 4 μl of dye (10% Ficoll/0.02% bromphenol blue/0.02% xylene cyanol) was added and the protein–DNA complexes were separated by electrophoresis in 4.5% polyacrylamide gels in low ionic strength buffer (22.5 mM Tris base/22.5 mM boric acid) at room temperature.

Binding reaction mixtures for DNase I protection experiments contained bovine serum albumin (500 μg/ml) in addition to the above components. After incubation at 20°C, 5 μl of appropriately diluted DNase I in buffer (20 mM Tris·HCl, pH 7.6/50 mM NaCl/1 mM dithiothreitol/15 mM $CaCl_2$/30 mM $MgCl_2$/100 μg of bovine serum albumin per ml) was added and incubation was continued for 1 min before ethanol precipitation. Digestion products were separated in sequencing gels (23).

Methylation interference binding reaction mixtures contained 200 μg of bovine serum albumin per ml and the DNA was partially methylated before binding (23). Bound and unbound fragment were separated by gel mobility shift, extracted from the gel, purified with an Elutip (Schleicher & Schuell), and subjected to piperidine cleavage (23). Cleavage products were separated in a sequencing gel.

Plasmid DNAs and Transfection Experiments. The pCAT-82SVSX, pCAT82SVSX-XBp, and pLAC82SU plasmids were described elsewhere (18). The oligonucleotides (see Fig.

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Abbreviations: nt, nucleotide(s); LTR, long terminal repeat.

3 legend) were cloned into *Sal* I sites in these plasmids. Orientation and number of inserts were determined by restriction and confirmed by sequencing in selected cases.

Transfections, heat shock, RNA preparation, Northern blots, and quantitation of transcripts were performed as described (18).

RESULTS

Previous experiments indicated that the repeat-containing sequence of gypsy increases the level of truncated transcript by increasing utilization of the LTR poly(A) site. When a LTR fragment [XB; *Xho* I/*Bgl* II fragment (nt 199–425) of the bx^{34e} gypsy element] containing the poly(A) site is placed in the intron of the *Drosophila hsp82* heat shock gene, some of the *hsp82* transcripts in transfected *Drosophila* cells are polyadenylylated in XB (18). The level of transcript polyadenylylated in XB is 5- to 10-fold higher when the 326-base-pair (bp) repeat-containing sequence (BaBx; nt 641–967), naturally located 351 nt downstream of the 5′ LTR poly(A) site in bx^{34e} gypsy (Fig. 1), is placed immediately downstream. Similarly, BaBx increases the level of RNA polyadenylylated at a weak poly(A) site in the *hsp82* intron (unpublished data). BaBx has no apparent effects on the levels of *hsp82* transcripts when placed upstream of either poly(A) site, indicating that it does not increase transcription. BaBx is effective in either orientation and does not contain palindromes longer than 4 bp. It seems unlikely, therefore, that it provides a signal in RNA. Because of these considerations, and the effects of *su(Hw)* mutations on 5′ LTR polyadenylylated transcripts, it was hypothesized that *Drosophila* nuclear extracts might contain proteins that bind the repeat in BaBx DNA.

***Drosophila* Nuclear Extracts Contain Protein That Binds Specifically to the Repeat Motif in BaBx.** Gel mobility shift assays (24, 25) were used to examine nuclear extracts (22) of cultured S2 *Drosophila* cells for proteins that bind BaBx DNA. Extract was incubated with labeled BaBx and the complexes were resolved by electrophoresis. The amount of BaBx bound by protein increased with extract concentration (Fig. 1*A*, lanes 2–4). Inclusion of pGEM-1 plasmid DNA in the reaction mixture reduced the amount of bound fragment, but two protein–BaBx complexes were still detected (lane 6). These complexes were not detected when pGEM-1 plasmid DNA containing BaBx sequences was included (lane 5), indicating that they resulted from specific binding.

The complex-forming abilities of fragments of BaBx containing different copies of the repeat sequence were examined. *Hin*cII cuts BaBx into two fragments, one (A) containing six copies of the repeat and one (B) containing two copies (Fig. 1). Both formed complexes (Fig. 1*C*, lanes 1 and 3). Furthermore, excess unlabeled B in the binding reaction prevented binding to labeled A (lane 4) and vice versa (lane 2), indicating that both bound the same factor. This suggests that the larger complex observed with BaBx may result from binding of factor to more than one site.

Sequences in the *Hin*cII fragments recognized by the binding activity were characterized by DNase I protection (26) and methylation interference (27) experiments. One

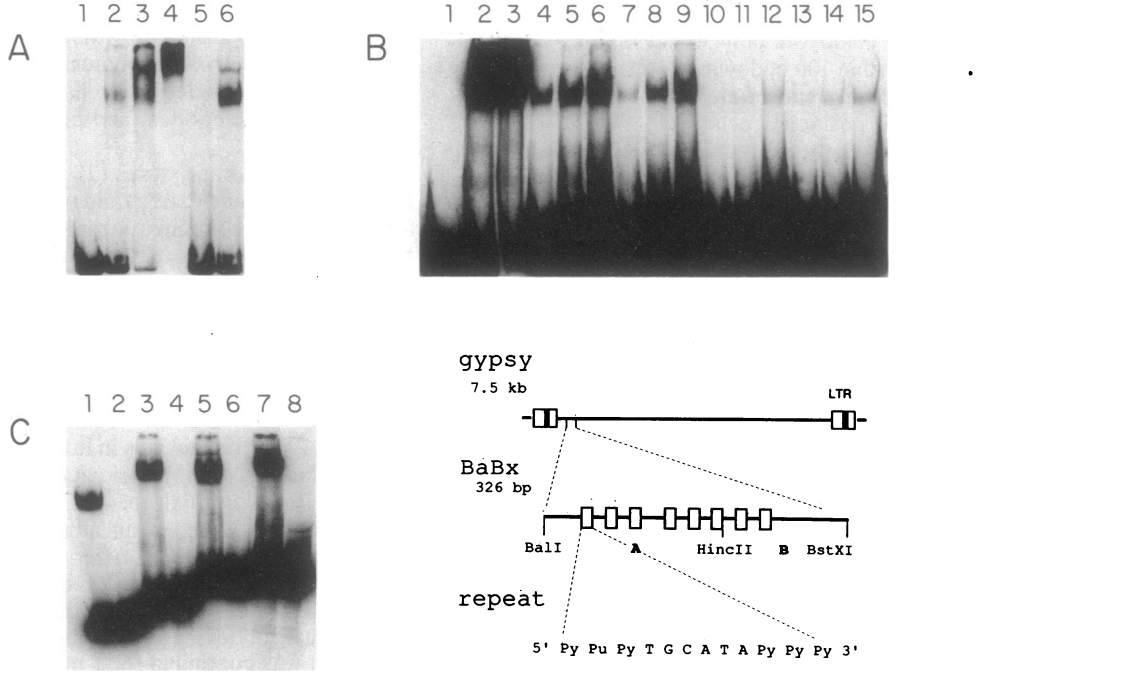

FIG. 1. *D. melanogaster* nuclear extracts contain BaBx-binding protein. Autoradiograms of gels used to separate BaBx fragment–protein complexes are shown. (*A*) Reactions with 0.16 μg of ^{32}P-labeled BaBx DNA per ml (bx^{34e} gypsy; nt 641–967), S2 cell nuclear extract, and different competitor DNAs. Lanes: 1, no extract; 2, 100 μg of nuclear extract protein per ml; 3, 200 μg of nuclear extract per ml; 4, 400 μg of nuclear extract per ml; 5, 200 μg of nuclear extract per ml and 20 μg of pGEM-1 plasmid with a BaBx insert per ml; 6, 200 μg of nuclear extract per ml and 20 μg of pGEM-1 per ml. (*B*) Reaction mixtures contained 0.16 μg of ^{32}P-labeled BaBx and various nuclear extracts per ml. Lanes: 1, no extract; 2 and 3, 400 and 800 μg of S2 extract per ml; 4–6, 200, 400, and 800 μg of Oregon R pupal extract per ml; 7–9, 200, 400, and 800 μg of $y^2\ w^a\ ct^6\ f^1$ pupal extract per ml; 10–12, 200, 400, and 800 μg of $y^2\ w^a\ ct^6\ f^1$;$su(Hw)^2/su(Hw)^f$ pupal extract per ml; 13–15, 200, 400, and 800 μg of $y^2\ sc^1\ v^1$;$su(Hw)^{f3}$ pupal extract per ml. Complexes were quantitated with a scintillation counter. (*C*) Reaction mixtures contained 200 μg of S2 nuclear extract per ml and various gypsy fragments. Lanes: 1, 0.06 μg of ^{32}P-labeled *Hin*cII B BaBx fragment per ml (bx^{34e} gypsy; nt 835–967); 2, ^{32}P-labeled *Hin*cII B and 2 μg of unlabeled *Hin*cII A fragment per ml (bx^{34e} gypsy; nt 641–835); 3, 0.1 μg of ^{32}P-labeled *Hin*cII A fragment per ml; 4, 0.1 μg of ^{32}P-labeled *Hin*cII A per ml and 2 μg of unlabeled *Hin*cII B per ml; 5, 0.16 μg of ^{32}P-labeled BaBx per ml; 6, 0.16 μg of ^{32}P-labeled BaBx per ml and 2 μg of unlabeled *Hin*cII A per ml; 7, 0.16 μg of ^{32}P-labeled BaBx per ml and 2 μg of unlabeled *Bgl* II/*Bal* I fragment per ml (with bx^{34e} gypsy; nt 425–641); 8, 0.16 μg of ^{32}P-labeled BaBx per ml and 2 μg of unlabeled *Hin*cII B fragment per ml. Internal gypsy sequences are represented by a line and LTRs are indicated by boxes. The 3′ end of the solid portion of the LTR is the poly(A) site. Repeats in BaBx are shown as open boxes.

region of each fragment was protected from DNase I digestion upon incubation with extract (Fig. 2 *A* and *D*). Both footprints were centered over a repeat and were of similar size (32–33 nt on the sense strand and ≈47 nt on the antisense strand). Methylation of any G residue within the consensus repeat or in the sense strand 5–7 nt 3′ to the repeat interfered with binding (Fig. 2 *B*, *C*, *E*, and *F*). These observations demonstrate that the repeat contains at least part of the recognition sequence for the binding activity. Footprints covering two other copies of the repeat in the *Hin*cII A fragment were observed with high amounts of extract (data not shown).

BaBx-Binding Activity Is Reduced in Pupae with *su(Hw)* Mutations. If the BaBx-binding activity is in part responsible for the effect of the BaBx region on the level of transcript truncated in the gypsy 5′ LTR, then the amount of binding activity might be reduced by the *su(Hw)* mutations that reduce the level of LTR-truncated transcript (17, 18). Indeed, in several independent experiments, the specific activity of BaBx-binding activity in nuclear extracts prepared from pupae of two fly stocks with mutant *su(Hw)* alleles [$y^2\ w^a\ ct^6\ f^1$;$su(Hw)^2/su(Hw)^f$, and $y^2\ sc^1\ v^1$;$su(Hw)^{f3}$; Fig. 1*B*, lanes 10–15] was 4- to 6-fold lower than in extracts from stocks with wild-type *su(Hw)* alleles (Oregon R and $y^2\ w^a\ ct^6\ f^1$; Fig. 1*B*, lanes 4–9). Pupae were used because effects of *su(Hw)* mutations on the level of LTR-truncated transcript have been observed at this stage (18). Complexes were formed under the same stringent binding conditions with pupal and S2 extracts, and the complexes formed with pupal extracts migrated with the faster-migrating complex formed with S2 extract (Fig. 1*B*; data not shown), suggesting that the factors in the cultured cell and pupal extracts were likely to be the same.

It was reported that the *su(Hw)* gene product binds to a restriction fragment overlapping BaBx (28). It is possible, therefore, that the repeat-binding factor detected here is a *su(Hw)* gene product. Because the *su(Hw)* alleles are not necessarily null (29), it would not be unexpected for the mutant extracts to have activity.

A Change in the BaBx Repeat Motif Reduces Protein Binding and Potentiation of a Poly(A) Site. To verify that potentiation of an upstream poly(A) site by BaBx results from binding of protein to the repeats and not an unrelated property, two 46-bp double-stranded oligonucleotides, differing in their ability to bind protein, were tested for their ability to potentiate a poly(A) site. The wild-type "binding" oligonucleotide contained the repeat motif present in the strongest binding

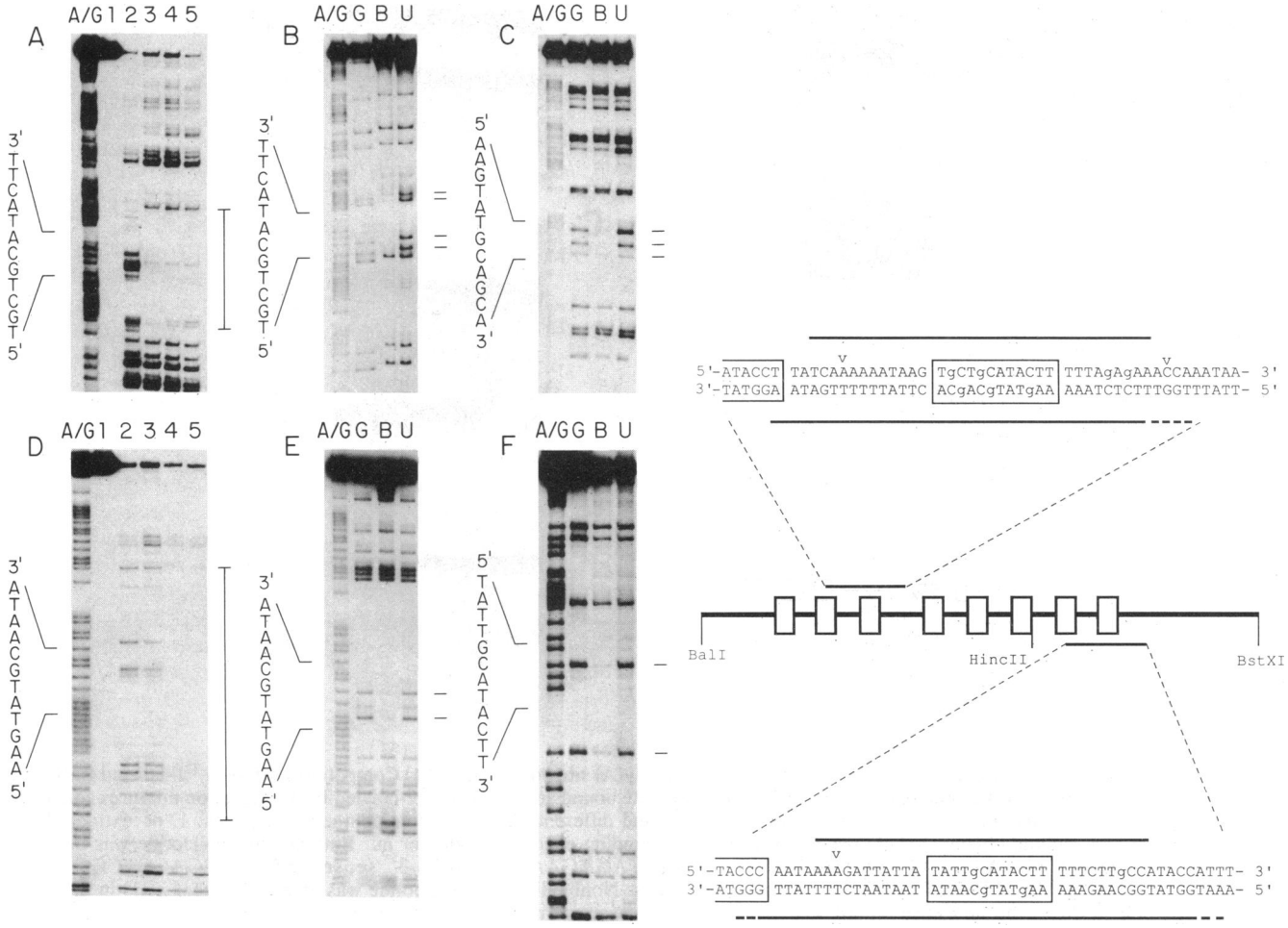

FIG. 2. The repeat motif is part of the recognition sequence for BaBx-binding factor. (*Left*) Autoradiograms of DNase I protection and methylation interference gels are shown. (*A*) DNase I protection analysis of binding to ^{32}P-labeled *Hin*cII A BaBx fragment (0.16 μg/ml) sense strand. Lanes: A/G, Maxam–Gilbert A+G ladder; 1, 50 μg of heparin-agarose purified extract per ml and no DNase I treatment; 2–5, 0, 25, 50, and 100 μg of heparin-agarose fraction per ml. (*B*) Methylation interference analysis of binding to *Hin*cII A fragment sense strand. Lanes: A/G, Maxam–Gilbert A+G reaction; G, Maxam–Gilbert G ladder; B, protein-bound fraction of partially methylated fragment; U, unbound portion. (*C*) Methylation interference with *Hin*cII A antisense strand; lanes are the same as in *B*. (*D*) DNase I protection of *Hin*cII B (0.1 μg/ml) antisense strand; lanes are the same as in *A*. (*E*) Methylation interference with *Hin*cII B antisense strand; lanes are the same as in *B*. (*F*) Methylation interference with *Hin*cII B sense strand; lanes are the same as in *B*. Results are summarized in the diagram (*Right*). Boxed sequences are the repeats; lines over or under the sequence indicate footprints; g, residues that interfere with binding when methylated; v, DNase I hypersensitive sites.

site in the BaBx *Hin*cII A fragment (TGCTGCATACTT), and the mutant "nonbinding" oligonucleotide was the same except for 1 bp (TGCTcCATACTT). Most of the sequence flanking the repeat was the same as in BaBx (legend to Fig. 3). The abilities of the oligonucleotides to bind protein were tested by competition assays. The binding oligonucleotide competed effectively with BaBx (Fig. 3*A*, lane 3), but the oligonucleotide with the altered repeat did not (lane 4).

The abilities of the oligonucleotides to potentiate an upstream poly(A) site were compared by cloning them into a modified *hsp82* gene containing the 226-bp *Xho* I/*Bgl* II gypsy LTR fragment (XB) in the intron (Fig. 3, CAT82SVSX-XBp; ref. 18). Since XB contains a poly(A) site, *hsp82* transcripts truncated in XB [1.2 kilobases (kb)] in addition to unspliced and spliced (1.8 kb) transcripts are present in RNA from S2 cells transfected with this gene (Fig. 3 *B* and *C*, lanes 3). The amounts of truncated transcript from genes containing different numbers of oligonucleotide insertions at the downstream end of the XB segment (in the *Sal* I site; Fig. 3) were compared by Northern analysis, relative to a transcript of a cotransfected *hsp82* gene with a different marker (LAC82SU; ref. 18). A gene with one binding oligonucleotide gave the same level of truncated transcript as the gene without oligonucleotides, relative to the control (Fig. 3 *B–D*, lanes 3 and 4; Fig. 4). Multiple tandem insertions, however, gave higher levels of truncated transcript. Genes with three copies (Fig. 3*C*, lane 5; Fig. 4) gave ≈3-fold more than the gene without oligonucleotides (lane 3). Seven copies of the binding oligonucleotide increased the level of truncated transcript 6- to 9-fold (Fig. 3*C*, lane 6; Fig. 4). In contrast, a gene with six copies of the nonbinding oligonucleotide (Fig. 3*C*, lane 7; Fig. 4) gave only 2-fold more truncated transcript than the gene without oligonucleotides. Six nonbinding insertions consistently gave 3- to 4-fold less truncated transcript than seven binding oligonucleotide insertions in all experiments. In addition, genes with fewer than six nonbinding oligonucleotide insertions gave less truncated transcript than genes with similar numbers of binding oligonucleotide insertions (Fig. 4).

The gene with seven binding oligonucleotides (Fig. 3 *B–D*, lanes 6) reproducibly gave 2- to 3-fold less spliced 1.8-kb

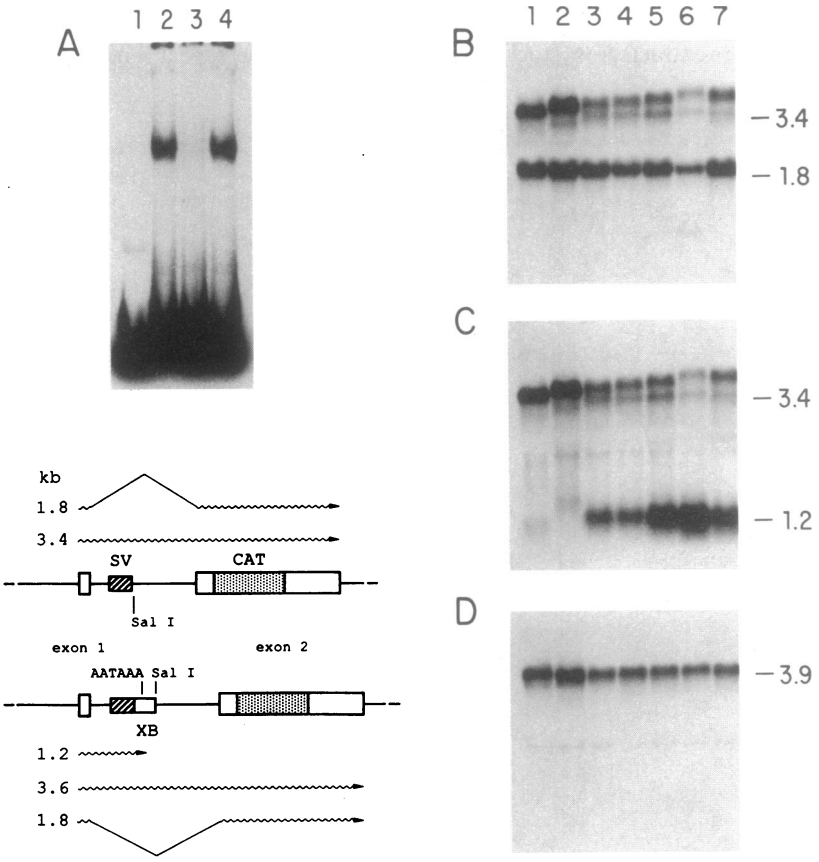

FIG. 3. A mutation in the repeat motif reduces protein binding and poly(A) site potentiation. (*A*) Competition between ^{32}P-labeled BaBx and oligonucleotides for binding. The autoradiogram is of a gel used to separate bound and unbound ^{32}P-labeled BaBx. Reaction mixtures contained 0.16 μg of BaBx DNA per ml, 100 μg of S2 nuclear extract per ml, and different oligonucleotide competitors. Lanes: 1, no extract; 2, no competitor; 3, 2 μg of binding oligonucleotide per ml; 4, 2 μg of nonbinding oligonucleotide per ml. Binding oligonucleotide was made by hybridizing 5′-TCGAGCTCAAAAAATAAGTGCTGCATACTTTTTAGAGAAACCGACG-3′ sense and 5′-TCGACGTCGGTTTC-TCTAAAAAGTATGCAGCACTTATTTTTTGAGC-3′ antisense strands. Nonbinding oligonucleotide was identical except nt 23 in the sense strand was C, and nt 28 in the antisense strand was G. (*B–D*) Transcripts of modified *hsp82* genes containing binding and nonbinding oligonucleotide insertions in transfected S2 cells. Autoradiograms are of the same Northern blot hybridized to CAT (*B*), SV (*C*), and LAC (*D*) probes. CAT probe detects spliced (1.8 kb) and unspliced (3.4 kb) CAT82SVSX transcripts, SV detects CAT82SVSX unspliced precursor and the truncated transcript of CAT82SVSX-XBp (1.2 kb), and LAC detects the 3.9-kb spliced LAC82SU transcript (30). Lanes contained 10 μg of total cellular RNA from heat-shocked (34.5°C for 20 min) cells. Cells were cotransfected with pLAC82SU control plasmid (1 μg/ml) and one of several pCAT82SVSX constructs (2 μg/ml). Lanes: 1, pCAT82SVSX with no oligonucleotide insertions; 2, pCAT82SVSX with five tandem insertions of binding oligonucleotide in the *Sal* I site (see diagram); 3, pCAT82SVSX-XBp (pCAT82SVSX with the gypsy LTR fragment in the intron; see diagram); 4–6, pCAT82SVSX-XBp with one, three, and seven tandem insertions of binding oligonucleotide; 7, pCAT82SVSX-XBp with six nonbinding insertions. Transcript sizes are in kb. Transcripts were quantitated as described (18). The CAT82SVSX and CAT82SVSX-XBp *hsp82* genes are shown in the diagram. Tall open boxes, *hsp82* exon sequences; lines, 5′ and 3′ flanking and intron sequences; stippled box, CAT sequence; hatched box, SV sequence in the intron. The gypsy XB LTR fragment containing the poly(A) site is shown as an open box downstream of SV. Transcripts are represented by wavy lines.

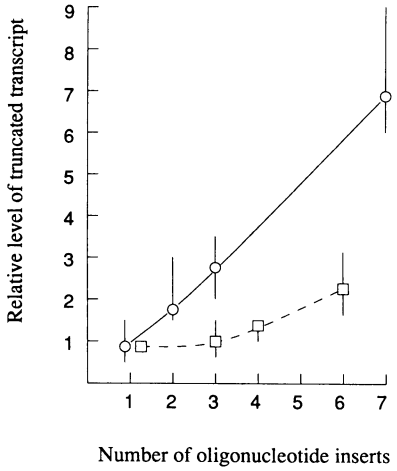

FIG. 4. The level of truncated transcript depends on the number of wild-type or mutant binding sites. Levels of truncated transcript produced by CAT82SVSX-XBp genes with various numbers of binding or nonbinding oligonucleotide insertions were measured by Northern analysis as in Fig. 3 and are expressed relative to the level produced by the CAT82SVSX-XBp gene without inserts. Each point is the average of three to seven independent experiments and vertical bars indicate the range of experimental values. ○, Levels obtained with the protein-binding oligonucleotide; □, levels obtained with the mutant oligonucleotide.

read-through transcript than the gene without oligonucleotides (lanes 3), indicating that the effect on the level of truncated transcript was unlikely to result from an increase in transcription. Furthermore, no effect on unspliced or spliced transcript levels was observed when five copies of the binding oligonucleotide were inserted into the *hsp82* gene in the absence of an XB segment (Fig. 3, lanes 2). The greater ability of the binding oligonucleotide to potentiate an upstream poly(A) site, therefore, confirms that BaBx potentiates poly(A) sites at least in part because of binding of protein to the repeat motif.

Although the mutant oligonucleotide did not detectably bind protein *in vitro*, it still slightly increased the amount of truncated RNA. Although it cannot be ruled out that some protein binding to the mutant sequence occurs *in vivo*, it is also possible that the repeat, or flanking A+T-rich sequences, have an intrinsic ability to influence use of poly(A) sites that is enhanced by protein binding. In either case, the 3- to 4-fold difference between the mutant and wild-type oligonucleotide is quantitatively similar to the observed effects of *su(Hw)* mutations on levels of gypsy-truncated transcripts (17, 18).

DISCUSSION

The results presented indicate that binding of protein to DNA in an internal region of the gypsy retroposon (BaBx) is involved in the potentiation of upstream poly(A) sites by this region. One can imagine more than one way in which a protein binding to DNA could influence utilization of an upstream poly(A) site. For example, it could pause or terminate transcription and thereby force greater utilization, or it could interact with the nascent transcript and influence RNA processing. The former is consistent with the observation that BaBx increases utilization of only upstream poly(A) sites. Furthermore, there are both prokaryotic and eukaryotic examples of DNA-binding proteins that can pause or terminate transcription. The *lac* repressor terminates transcription of its own gene (30). Proteins binding to the mouse and *Xenopus laevis* rRNA gene promoters terminate transcription by RNA polymerase I (31–33). A CCAAT box region in the adenovirus major late promoter has been observed to pause or terminate RNA polymerase II transcription (34). Because a small 13-bp segment containing a CCAAT box protein-binding site is necessary and sufficient (35), it has been suggested that protein binding mediates termination.

Because gypsy is a transposon and can insert downstream of a variety of promoters, one can speculate that one of the functions of the BaBx repeat motif is to increase use of the 5′ LTR poly(A) site and thereby decrease inadvertent expression of gypsy by transcription initiated upstream. Although the mechanism of poly(A) site potentiation is unknown, the evidence presented clearly indicates involvement of a DNA-binding protein.

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