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Differential Binding of the *Bombyx* Silk Gland-Specific Factor SGFB to Its Target DNA Sequence Drives Posterior-Cell-Restricted Expression

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The gene encoding the silk protein P25 in *Bombyx mori* is expressed in the posterior silk gland (PSG) cells and repressed in the middle silk gland (MSG) cells. To identify the factors involved in this transcription-dependent spatial restriction, we examined the *P25* chromatin in PSG and MSG nuclei by DNase I-aided ligation-mediated PCR and analyzed the expression of various *P25-lacZ* constructs in biolistically treated silk glands. *P25* promoter activation depends on two *cis*-acting elements. One coincides with the target sequence of SGFB, a silk gland-specific factor present in all silk gland nuclei, but bound to its target DNA sequence in only PSG cells. The interaction of the other element with a factor that we named PSGF is also exclusive to PSG cells. Placed ahead of a non-P25-related basal promoter, the SGFB and PSGF elements are sufficient to drive posterior-cell transcription. Collectively, our data support the hypothesis that the spatial restriction of P25 expression is driven by the stabilization of SGFB onto its target sequence by the action of PSGF.

Organogenesis proceeds by selective gene activation and repression, resulting in the appropriate complement of trans-regulatory factors that define the different cell types. Identifying these factors that govern each category of cell is one step toward understanding gene selection during embryogenesis. Silk gland organogenesis in *Bombyx mori* is completed in the embryo as evidenced by the spatial organization of fibroin- and sericin-secreting cells in, respectively, posterior and middle positions (2). As a consequence, the five single-copy genes encoding the various silk proteins of *Bombyx* are expressed in the embryo in distinct groups of the ~750 cells that constitute the silk gland epithelium.

One such gene encodes the protein P25, a silk polypeptide that binds to the heavy and light subunits of fibroin. This gene is highly expressed in the posterior silk gland (PSG) cells and repressed in the middle silk gland (MSG) cells (3, 4, 30).

To understand the regulation of *P25* expression, an in vivo analysis was performed with transgenic *Drosophila melanogaster*. These studies demonstrated that the *Bombyx* promoter is regulated by *Drosophila trans*-acting factors in the salivary gland, the silk gland homolog in the fruit fly (1). As in the *Bombyx* silk gland, the activity of the *P25* promoter in the salivary gland is positionally determined and is controlled by upstream sequences located within the 441 bp of proximal DNA.

To identify putative regulatory elements, in vitro DNA-protein interactions between silk gland nuclear factors and synthetic DNA probes were explored, and two possible regulators with different properties were discovered (5). The first, BMFA, is a ubiquitous protein thought to be involved in the repression of silk gland-expressed genes at molting, including those encoding silk proteins. The second, SGFB, is a silk gland-specific regulatory protein expressed in both PSG and MSG cells and thus unable, by itself, to specify PSG expression. Three other proteins (TRIO, Ub2a, and Ub2b) that bind to probes derived from *P25* 5′ flanking sequences have also been characterized in vitro, but all were expressed in various larval tissues throughout development (24).

To understand the function of the factors involved in PSG-specific expression, we combined two novel approaches to study *P25* chromatin structure and *P25* in vivo activation in fully differentiated silk glands. We report that the selective expression of *P25* in PSG cells is controlled by two factors, SGFB and PSGF, a novel regulatory protein. Although present in all silk gland cells, SGFB binds, or has access to, its target sequence only in PSG cells. The implications of these results for understanding the molecular mechanisms of silk protein gene regulation are discussed.

MATERIALS AND METHODS

Animals. *Bombyx* larvae were F_1 hybrids of the European strains 200 and 300. They were reared either with fresh mulberry leaves at 23°C or on an artificial diet at 25°C.

In vivo footprinting. In vivo chromatin footprinting was carried out by a two-step protocol whereby silk gland nuclei were treated with DNase I and the DNA region of interest was amplified by ligation-mediated PCR (LMPCR) (22).

(i) In situ DNase I treatment. A new procedure for digestion of chromosomal DNA with DNase I was developed. Typically, six PSGs or nine MSGs dissected from larvae at day 3 of the fifth instar were frozen and powdered under liquid nitrogen with a mortar to break the nuclear lobes (at the stage analyzed, each silk gland cell nucleus contains 2×10^5 to 4×10^5 haploid genome equivalents). DNase I partial digestion was performed by incubating the powdered tissues for 15 min at room temperature in 3 ml of a buffer containing 35 mM HEPES (pH 7.4), 150 mM sucrose, 80 mM KCl, 5 mM K_2HPO_4, 5 mM $MgCl_2$, 2 mM $CaCl_2$, and 25 μg of DNase I (Boehringer Mannheim Biochemicals) per ml. The reaction was stopped by the addition of 3 ml of 20 mM Tris-HCl (pH 8.0)–20 mM NaCl–20 mM EDTA–1% sodium dodecyl sulfate–10 μg of proteinase K per ml. After overnight incubation at 37°C, DNA was purified and ethanol precipitated as described previously (25).

Genomic DNA (80 μg/ml) was resuspended in the same buffer as described above and cleaved with DNase I (20 ng/ml) for 1 min at room temperature.

(ii) LMPCR. Amplification of *P25* DNA fragments generated by DNase I treatment was carried out essentially as described by Garrity and Wold (7). Genomic DNA (3 to 5 μg) was treated successively with Sequenase (U.S. Biochemical Corporation) and Vent DNA polymerase (New England Biolabs, Inc.). Exponential amplification and labelling reactions were carried out in 20 and 3 cycles, respectively. The labelled amplified products were phenol chloroform extracted, ethanol precipitated, and resuspended in a formamide-dye mixture prior to separation in an 8% polyacrylamide-urea gel. Primer sequences, their locations relative to the *P25* transcription start site, and hybridization tempera-

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tures used to analyze the *P25* regulatory regions on both strands were as follows for the coding strand: P1, 5′-AGCATGTTGCGCGAATAATAAT-3′ (+9 to +30), 50°C; P2, 5′-AAACAACTGCGTCGTTAGGC-3′ (−12 to +8), 56°C; and P3, 5′-CTGCGTCGTTAGGCACTGATCAAC-3′ (−22 to +2), 59°C. For the noncoding strand these parameters were as follows: P1, 5′-CAGTTACTGCCA CACAAAC-3′ (−203 to −185), 50°C; P2, 5′-TTAACTCCCCGCCTACGTCG A-3′ (−180 to −160), 56°C; and P3, 5′-CGTCGAGGAGAACATTTTGCGCC T-3′ (−165 to −142), 59°C.

Plasmid construction. A (*−1451*)*P25-lacZ* fusion gene was constructed by fusing the *P25* DNA from position −1451 to +26 (which includes the *P25* ATG initiation codon) to the β-galactosidase-coding region at codon 8. The 3′ untranslated region of the fusion gene was that of the cytoplasmic actin *A3* gene (10). All fusion genes carrying deletions or internal mutations were cloned into the same plasmid, in the same relative orientation.

(i) 5′ recurrent deletions. Deletion mutants were generated by BAL 31 digestion of (*−1451*)*P25-lacZ*. The endpoints of digested DNA molecules were determined to be at positions −437, −319, and −251 by standard dideoxy sequencing.

(ii) Internal mutations. The mutations in the Ub2a, Ub2b, BMFA, and SGFB elements which abolish the binding of their cognate factors (5, 24) were created by ligating appropriate fragments resulting from 5′ and 3′ BAL 31 digestions of *P25* DNA. The wild-type sequences were replaced by the same exact number of nucleotides taken from the plasmid polylinker DNA. All other mutations described in Fig. 3 were generated by PCR-mediated mutagenesis according to the protocol of Schier and Gehring (28), using different sets of mutated and wild-type primers.

(iii) Synthetic *P25-A3* mixed promoter. The target sequences of the factors SGFB and PSGF were inserted upstream from the TATA box of the *Bombyx A3* cytoplasmic actin gene in a (*−30*)*A3-lacZ* fusion gene. The two motifs were introduced by PCR-mediated insertion with respect to their relative positions in *P25* DNA. The chimeric promoter sequence was as follows:

...ctcgag**CTATTTATTTAACG***acgcaaataaatt***GGAACAATACTTT**tata<u></u>tagttt..
*Xho*I SGFB random PSGF *A3* sequence

The *SGFB-PSGF*(*−30*)*A3-lacZ* fusion gene was cloned at an *Xho*I site in plasmid DNA.

Biolistic assay. DNA was introduced into silk gland cells by using a particle delivery system (PDS-1000/He; Bio-Rad) (11). Pressurized helium (400 lb/in^2) was used to propel DNA-coated tungsten particles (average size, 1.8 μm) onto silk glands freshly dissected from larvae at day 3 of the fifth instar. The distance to the target was 12 cm. DNA (2.5 to 15 μg per assay) was precipitated onto the microprojectiles in 1 M $CaCl_2$–0.016 M spermidine as previously described (27). The microprojectiles were pelleted and gently resuspended before being mounted, without dehydration, into a modified launch assembly, as described previously (20). After bombardment, the organs were returned to Grace's medium and rapidly reimplanted into host larvae taken at day 4 of the fifth instar. One silk gland was introduced per larva. The grafted animals were reared on mulberry leaves or an artificial diet for 48 h. At this time, the transplant was dissected out, and the expression of the gene construct was assayed in toto by X-Gal (5-bromo-4-chloro-indolyl-β-galactosidase) staining (9). Quantitation of promoter activity was carried out by bombarding simultaneously 5 μg of *P25-lacZ* and 5 μg of *A3-CAT* used as an internal reference (*A3* is the promoter of the *Bombyx* cytoplasmic actin gene *A3*; *CAT* encodes chloramphenicol acetyltransferase [CAT]). β-Galactosidase activity in homogenates from treated and nontreated silk glands was assayed as described by Rosenthal (26). CAT activity was measured by using quantitative kinetic data (23) with a fraction of the same silk gland homogenates.

RESULTS

Differential chromatin footprinting of *P25* in PSG and MSG. In order to characterize the chromatin organization of *P25* in PSG and MSG cells, we examined the pattern of DNase I partial digestion of native chromatin and naked genomic DNA by means of LMPCR amplification of digested fragments (22). A new method of DNase I treatment was developed, in which the enzyme is directly applied to liquid-nitrogen-crushed PSG or MSG giant nuclear lobes. The high content of haploid genomes per silk gland cell (2×10^5 to 4×10^5) allowed the preparation of large quantities of cleaved chromatin DNA from only six PSGs or nine MSGs. DNase I cleavage followed by heat denaturation resulted in single-stranded DNA with a size range of ~500 to 4,000 nucleotides. Highly reproducible patterns were obtained after LMPCR processing, showing clear differences between chromatin derived from PSG and MSG cells.

The differences in accessibility to the nuclease detected in the region extending from position −130 to −20 with the primers shown at the top of Fig. 1A are summarized in Fig. 1B for both categories of cells.

The DNase I cleavage pattern of the TATA-box region of the PSG nuclei was characterized by a strong protection of nucleotides at positions −33, −32, and −31 on the noncoding strand, suggesting the presence of TBP. No modification was observed on the coding strand. The comparison of PSG and MSG chromatin to naked genomic DNA revealed an MSG-specific hypersensitivity at the adenine at position −28 on the coding strand just downstream of the presumably inactive *P25* TATA box.

Immediately upstream, a region with marked differences between PSG- and MSG-derived chromatin was found. This region consisted of clustered PSG-specific hyperreactive nucleotides at positions −44, −43, −42, −39, and −37 on the coding strand and of protected positions at −45, −44, −42, and −41 on the noncoding strand. On the same strand, the cytidine at position −50 was found to be strongly hypersensitive. Such alterations of DNase I accessibility were clearly PSG specific, since MSG chromatin did not show significant and reproducible differences from naked genomic DNA. This revealed the presence in PSG chromatin of a novel factor which we designated PSGF (for PSG factor).

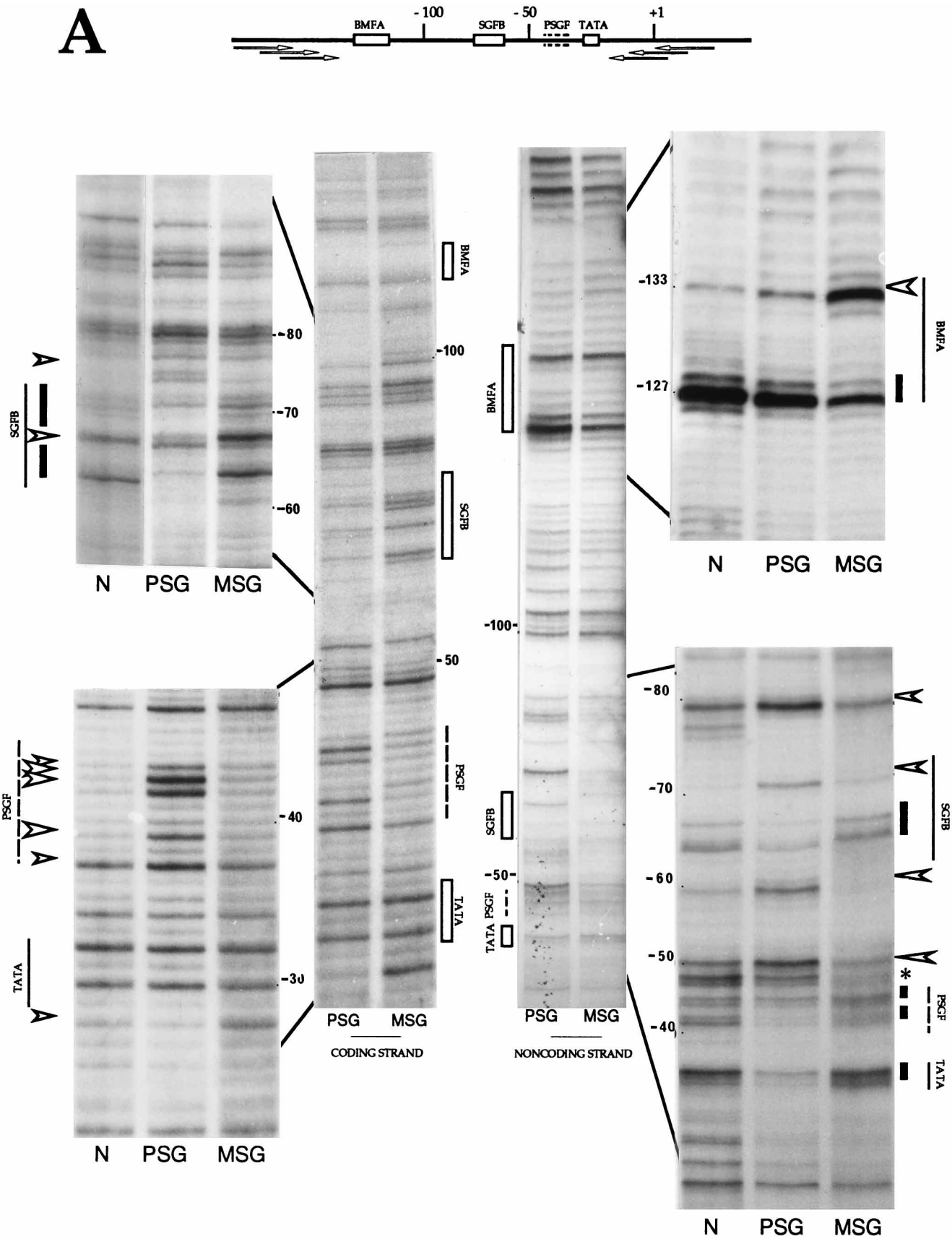
Further upstream, a second region encompassing the SGFB target site displayed cleavage alterations that were also characteristic of PSG chromatin. These included protection of the ATTT tandem motifs as observed in vitro (5) at positions −71 to −68 and −64 to −66 on the coding strand. The adenine at position −67 of the second motif and the upstream adenine at position −76 were found to be hypersensitive. The opposite strand was characterized by a protection over the four nucleotides from positions −68 to −65. These protected regions were flanked by two hypersensitive residues: a thymine, included in the SGFB target sequence at position −71, and an adenine at position −59. The thymine at position −79 was also systematically overcleaved. These observations demonstrated that SGFB is bound to its target sequence in PSG nuclei. In contrast, the same factor induced no footprint in MSG chromatin.

Of interest, differential DNase I cleavages further upstream were less frequent and coincided with the BMFA binding site. The cleavage sites were characterized by protection of the two neighboring cytosines at positions −128 and −127 and a strong hypersensitivity at the thymine at position −133 on the noncoding strand. No footprint was observed on the coding strand.

Three additional sets of primers were used to span the more upstream flanking DNA from position −260 to −130 on both the coding and the noncoding strands. Surprisingly, no difference was observed between PSG, MSG, and naked genomic DNA, except for a 3-nucleotide hypersensitive site centered at position −202 (results not shown). Sequences motifs for TRIO, Ub2a, and Ub2b showed no differential DNase I sensitivity in PSG and MSG chromatin compared to naked genomic DNA. This suggests that these proteins do not play a significant role in *P25* stimulation during intermolt.

These results revealed clear PSG-specific DNase I cleavages clustered in the proximal upstream region of the *P25* DNA, suggesting that SGFB and an unknown factor, PSGF, are involved in gene activation. To examine whether these structural observations have functional significance, we tested a variety of *P25*-modified promoters in PSG and MSG cells by a novel in vivo assay.

Functional analysis of *P25* regulatory sequences. Biolistic bombardment of *P25-lacZ* constructs into silk gland cells was utilized to identify the regulatory sequences involved in PSG-


A
BMFA
-100
SGFB
-50
PSGF
TATA
+1
-80
-70
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SGFB
N
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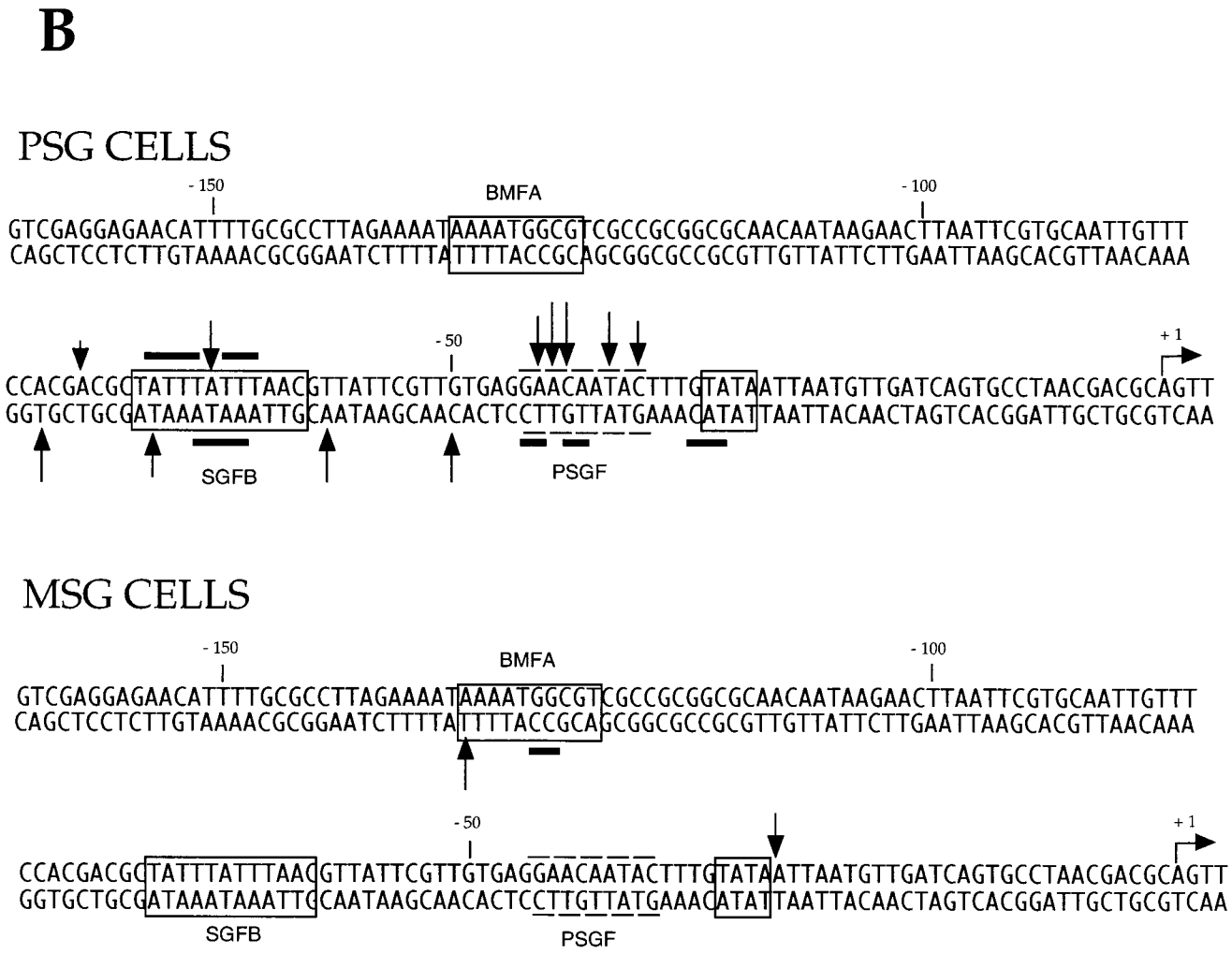

FIG. 1. DNase I genomic footprinting of the *P25* regulatory region. (A) *P25* coding (left) and noncoding (right) strands from PSG and MSG chromatin and from naked genomic DNA (N). The horizontal arrows at the top indicate the locations of the primers used for LMPCR. All gels have same gene orientation (5′ at the top). The positions of putative functional elements are indicated along the ladders. Arrowheads mark the nucleotides that are consistently hypersensitive to DNase I. Large arrowheads reflect enhanced hypersensitivities. Nucleotides protected from DNase I are shown by black boxes. The asterisk indicates apparent, but not reproducible, protection of the nucleotides at positions −48 and −47 in MSG nuclei. (B) Summary of positions with DNase I hypersensitivity or protection in the 5′ proximal *P25* DNA in PSG and MSG cells. All hyperreactive sites are indicated by arrows, the lengths of which reflect the degree of DNase I hypersensitivity. The protected stretches of DNA are shown by black boxes. The target sequences of the different factors were determined in vitro in previous studies (see text).

specific expression. DNA-coated tungsten particles were introduced into freshly dissected silk glands, and expression was monitored 48 h after the treated organs were grafted into recipient larvae. Promoter activity was analyzed by X-Gal staining of the treated silk gland and by semiquantitative measurement of β-galactosidase activity in PSG and MSG cells with reference to CAT activity driven by an *A3-CAT* fusion gene. This construct, in which the promoter of the *Bombyx* cytoplasmic actin gene directs the synthesis of the enzyme, was cobombarded with *P25-lacZ* constructs.

Episomal copies of the construct *P25-lacZ* harboring 1,451 bp of upstream *P25* DNA were correctly regulated, since expression occurred in PSG and not MSG cells (Fig. 2a and 3). As expected, the *A3-lacZ* construct used as a control was shown to drive β-galactosidase production in both PSG and MSG cells (Fig. 2i) whereas a promoterless *lacZ* sequence resulted in no β-galactosidase expression (not shown).

When the amount of DNA bombarded onto the silk gland was increased from 2.5 to 15 μg, the proportion of β-galactosidase-positive cells rose from 20 to 50% to nearly 100% of the cells (compare Fig. 2a and c). Thus, endogenous factors involved in *P25* promoter recognition were not rate limiting in the assay. In contrast with the response of PSG cells, not a single MSG cell displayed X-Gal staining, even following bombardment with large quantities of DNA.

By using 5′-deleted constructs, the domain capable of promoting high PSG-specific transcription was restricted to the 251 bp upstream from the transcription start site (Fig. 2b, d, and e and Fig. 3). A series of fusion genes harboring a single defined deletion or base substitutions (either single or combined) were then tested. These mutations were made in sequences showing in vivo footprinting or in the presumptive *cis* elements delineated by previous in vitro analyses. The alteration of the TRIO, Ub2a, Ub2b, and BMFA target sequences was not accompanied by any apparent modification of expression of the β-galactosidase-encoding construct. This is consistent with the absence of footprinting on these sequences in PSG chromatin and reinforces the hypothesis that these ele-

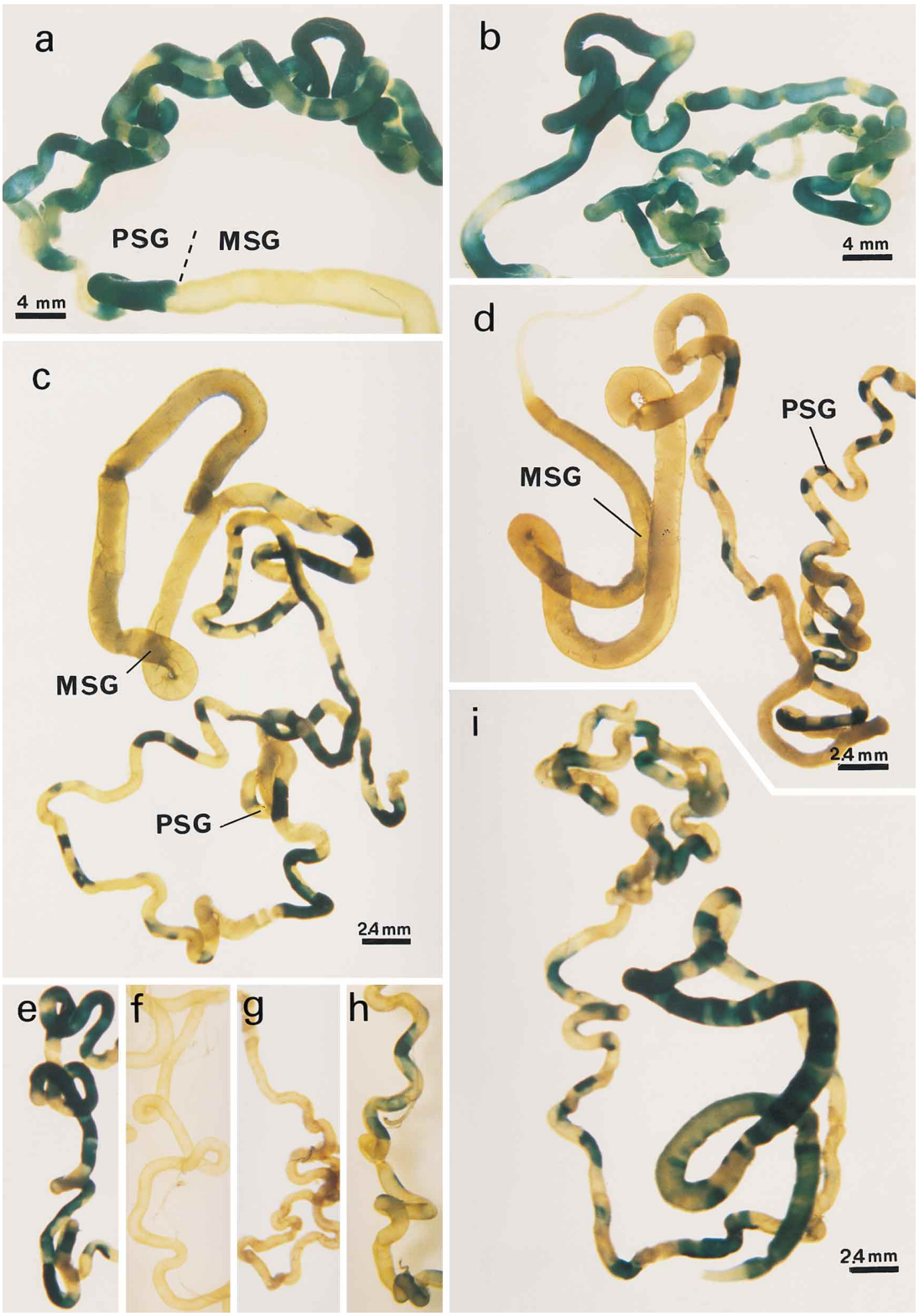

FIG. 2. Histochemical detection of β-galactosidase in silk glands reimplanted after biolistic transformation with *P25-lacZ* fusion genes. (a and b) Two silk glands bombarded with 15 μg of (*−1451*)- and (*−251*)*P25-lacZ* DNA, respectively. Note that almost all PSG cells are β-galactosidase positive with either construct. The sharp cell-to-cell boundary between positive PSG and negative MSG regions appears clearly in panel a. (c) Silk gland bombarded with 2.5 μg of the same construct as in panel a and harboring 32% positive PSG cells and no positive MSG cells. (d) Gland bombarded with 2.5 μg of the (*−437*)*P25-lacZ* deletion mutant, showing activity comparable to that in panel c. (e, f, g, and h) PSG fractions of glands treated with (*−251*)*P25-lacZ*, *mutSGFB*(*−251*)*P25-lacZ*, *mutPSGF*(*−251*)*P25-lacZ*, and *SGFB-PSGF*(*−30*)*-A3-lacZ*, respectively, at 10 μg/assay. Note the absence of activity in panels f and g and the presence of activity in h (although lower than in panel e). (i) The *A3-lacZ* fusion gene carrying the wild-type promoter of the cytoplasmic actin *A3* is expressed in PSG as well as in MSG cells.

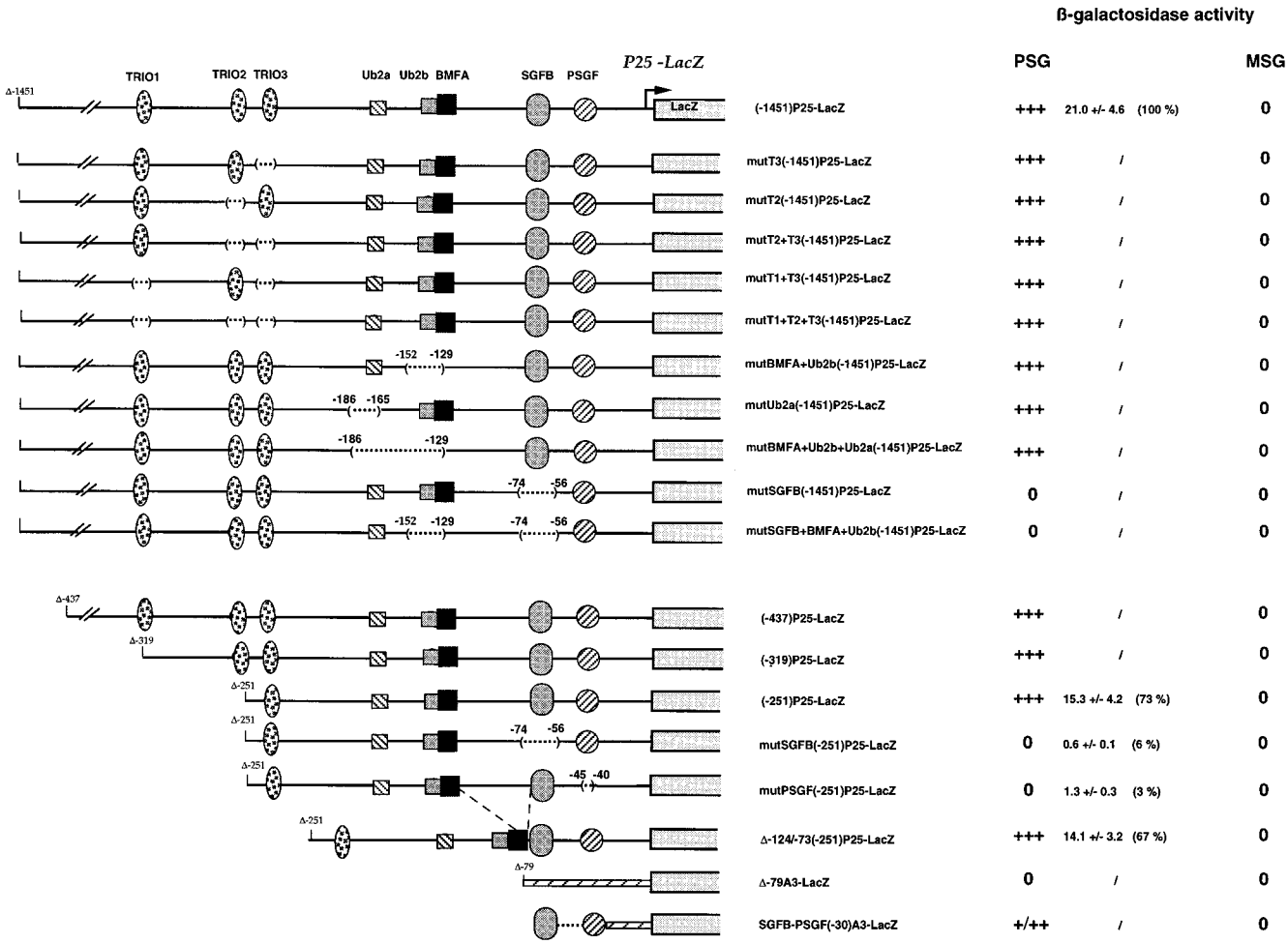

FIG. 3. Expression of *P25-lacZ* constructs in PSG and MSG cells. The recurrent deletions and mutations of the *P25* promoter used in the in vivo functional assay are shown. *P25* genomic DNA is shown as a thin line, whereas the *Escherichia coli* β-galactosidase-coding sequence is represented as a shaded box. The locations of the different target sequences of the putative functional factors are shown for all gene constructs. Deletion endpoints are indicated at the 5′ sites of fusion genes. Dotted lines represent the substituted DNA sequence. The borders of the mutated stretch are indicated above the constructs. The mutation in the TRIO elements corresponds to a 3-base substitution. The hatched box represent the cytoplasmic actin *A3* gene sequences. β-Galactosidase activity is indicated either by a qualitative assessment or by a semiquantitative index. Quantification is expressed in arbitrary units (β-galactosidase relative to CAT activities [see Materials and Methods]) as means ± standard deviations. Relative promoter activities are indicated in parentheses.

ments and their cognate factors are not involved in *P25* activation during the intermolt stage (Fig. 3).

In contrast, when the SGFB binding site was mutated in either the (*−1451*)- or the (*−251*)*P25-lacZ* construct, a strong effect was observed, with no β-galactosidase activity detected by in situ staining, even with 15 μg of DNA bombarded (Fig. 2f). Semiquantitative β-galactosidase measurements indicated that only 6% of promoter activity was retained when a mutation prevented SGFB interaction with the (*−251*)*P25-lacZ* promoter (Fig. 3). These results demonstrate that SGFB plays a key role in promoter activation.

The removal of DNA sequences between BMFA and SGFB, which includes the PSG chromatin-hypersensitive residues at positions −77 (coding strand) and −80 (noncoding strand) (Fig. 1B), had no strong effect on expression. Thus, the enhanced accessibility of DNase I at these positions is likely to result from distortions of DNA when SGFB binds to its target sequence.

In addition, a very strong negative effect was noted following mutation of the PSGF sequence, which showed opposite DNase I accessibilities in PSG and MSG chromatin. Substituting the hexanucleotide AACAAT (positions −44 to −39) for GTGAGG resulted in a drastic reduction to 3% of promoter activity and in an absence of β-galactosidase in situ staining (Fig. 2g and 3).

In an attempt to identify PSGF, retardation assays were developed, but no evidence was found of in vitro interaction of silk gland nuclear proteins with PSGF probes (extended to eventually include the SGFB target sequence) under a variety of reaction conditions (results not shown).

Taken together, our data on chromatin structure and silk gland cell expression converged to show that PSG activation of *P25* depends on both the SGFB and the PSGF elements. To directly demonstrate that these two elements are sufficient to promote PSG-specific expression, they were introduced upstream from an *A3* basal promoter fused to the *lacZ* coding sequence. The relative positions of the SGFB and PSGF target sequences in the chimeric promoter were the same as in native *P25* DNA (see Materials and Methods). As shown in Fig. 2h and 3, *SGFB-PSGF*(*−30*)*A3-lacZ* promoted β-galactosidase expression in PSG but not in MSG cells, whereas a (*−79*)*A3-lacZ* control DNA devoid of *A3* activating sequences (18) was

unable to drive β-galactosidase accumulation in bombarded silk glands. Although the activity of the chimeric promoter was less intense than that of *P25* (compare Fig. 2e and f), this showed that the SGFB and PSGF elements are sufficient to drive PSG-specific expression.

DISCUSSION

The use of chromatin footprinting proved invaluable in helping to distinguish PSG or MSG chromatin of the gene encoding the silk protein *P25*. By monitoring changes in accessibility to DNase I, sequences that might be involved in establishing the active or inactive state of the gene were delineated. The reproducibility of the cleavage patterns validates this rapid method, by which DNase I was applied directly to liquid-nitrogen-powdered silk gland cells. The efficiency of the procedure is clearly related to the homogeneity of the PSG and MSG cell populations, the very large size of their nuclear lobes, and their extremely high DNA content. With the biolistic reimplantation method, episomal constructs were assayed in fully differentiated silk gland cells maintained in their natural physiological environment. Faithful reproduction of the native conditions is demonstrated by the almost normal growth of the treated silk gland after reimplantation and by the differentially regulated activity of the *P25* promoter of the fusion gene in PSG and MSG cells, reflecting the normal transcriptional states of the endogenous gene.

We showed that a *P25-lacZ* construct with 251 bp of 5′ DNA is expressed in a cell-specific manner. Addition of various lengths of more-upstream DNA induced no qualitative changes and only a mild quantitative overstimulation. Cell-specific expression occurred despite the fact that the introduced genes were not integrated into chromosomes. Therefore, the creation of efficient chromatin structure in extrachromosomal genes relies on diffusible regulatory factors and structural proteins.

The integrity of two proximal *cis*-acting elements appears to be sufficient to achieve the high degree of specificity of *P25* expression in PSG cells. Unequivocally, one of these motifs corresponds to the SGFB binding element, since its alteration within the 1,451- or 251-bp upstream DNA led to almost complete inhibition of expression. This correlates with the footprint on the target DNA in PSG chromatin, strongly suggesting that the factor SGFB, a silk gland-specific protein, acts as a transactivator. In vitro characterization by gel retardation assay, proteolytic clipping, and SGFB-DNA cross-linking did not reveal structural differences between the SGFBs in PSG and MSG cells (5). However, our results clearly show that the silk gland factor stably binds to its target sequence in *P25*-active cells and not in inactive MSG cells.

The second element resides between the SGFB site and the TATA box. Accordingly, mutation of the underlying sequence drastically reduced expression. The hypothesis that a factor, designed PSGF, binds to this sequence in vivo is substantiated by the existence of DNase I protection and enhanced cleavage in PSG nuclei. Our previous in vitro explorations of the 440-bp upstream *P25* DNA to target PSG and MSG silk gland nuclear proteins failed to reveal precise protein binding at the corresponding sequence (5, 24). Recent reexaminations led to the same observation, reminiscent of that reported for other systems (8, 21). This may be due to a variety of parameters, such as scarcity and/or low-affinity binding of the regulatory protein to its target sequence or a requirement for conformational constraints of DNA that are not reproduced in vitro.

The observation that the SGFB and PSGF elements are sufficient to drive PSG-specific expression, when placed ahead of an *A3*-derived basal promoter, reinforces the decisive role of their cognate factors in the production of specific silk protein in posterior cells. The synthetic promoter was less active, however, than that of *P25*, which may indicate that the 251-bp upstream sequence of the gene contains an activating element that remains to be identified.

Consistent with our results, PSGF could play a role in stabilizing the SGFB-DNA complex on the *P25* PSG promoter. This dual action fits the current view of the manner by which gene transcription is regulated (reviewed in reference 31) but raises the question of the relationship between the two factors.

PSGF could facilitate the recruitment of SGFB and may represent an early event in the building up of the active transcription complex. Such a synergistic effect, whereby the binding affinity (or half life) of DNA-protein complexes is enhanced upon the binding of a neighboring factor, has been reported for several systems in vitro (14, 35). For example, Wright et al. (34) reported in vivo NFY-mediated stabilization of X-box factors in the human HLA-DRA gene. The effect was constrained by proper stereospecific alignment between NFY and the X-box factors, suggesting that they interact. The short distance between the SGFB and PSGF sites makes it very plausible that the factors establish contact. However, the topology seems not to have strict functional specificity, since the insertion of four nucleotides between the two sites has no significant effect on PSG expression (result not shown).

A specific feature of MSG chromatin is the footprint at the BMFA binding site. Our observation that the deletion of the corresponding element did not derepress gene expression in MSG cells demonstrates that the inactive state of *P25* arises from the absence of a factor with activating properties. Our data support the hypothesis that the factor missing in MSG cells is PSGF itself. However, the BMFA target site that showed differential specific alteration in MSG chromatin has been described as a potent repressor (5), and it is therefore possible that *P25* inhibition results from a combination of both mechanisms, the absence of PSGF and the attachment of BMFA to DNA.

As with other silk protein-encoding genes, *P25* is periodically turned on and off in PSG cells, during the succession of molts and intermolts. This cycle is accompanied by profound remodeling of chromatin structure, as revealed by studies on the H-fibroin gene (16, 33). Growing evidence suggests local unfolding as a determinant of gene activation (19). For example, nucleosome disruption mediated by the binding of a GAGA factor appears to be a prerequisite for the construction of the *Drosophila* heat shock gene transcription complex (32). The study of *P25* repression and derepression at the transitions between molts and intermolts would help in understanding the relationship between SGFB and PSGF and the mechanism that leads to nucleosomal rearrangement.

SGFB involvement in PSG-specific *P25* activation could probably be extended to other silk protein-encoding genes. This is suggested by the presence of a canonical SGFB element in both *H-Fib* and *L-Fib*, the PSG-specific genes that encode the heavy and light fibroin chains to which *P25* binds (13, 15). This is reinforced by the observation that the so-called OBF1 element (identical to SGFB) activates in vitro transcription of *H-Fib* in a silk gland system (12, 29). The contribution of the newly described PSGF to PSG-specific gene expression is also suggested by the presence in the *H-Fib* and *L-Fib* proximal promoters of heptanucleotide motifs (GGttCAA and GGAA CAt) closely related to the PSGF element (GGAACAA).

Interestingly, the restriction of gene expression in PSG cells appears to be similar to that in MSG cells, where the silk protein gene *Ser-1* expression depends also on two distinct

factors: a POU-like, MSG-specific factor (6) and a Forkhead/HNF3-like factor not specific to MSG cells (17).

Our study provides a novel explanation for PSG cell-specific gene expression. The availability of SGFB, the purification of which is currently in progress, will allow studies on the molecular cross-talk between the two regulatory proteins that control PSG-specific transcription.

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B. Horard and E. Julien contributed equally to this work.

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