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Genetics

Analysis of a DNase I-hypersensitive site in transgenic *Drosophila* reveals a key regulatory element of *Sgs3*

(glue gene/oligonucleotide-directed mutagenesis/tissue-specific gene expression)

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ABSTRACT **We have undertaken chromatin studies on transformed *Drosophila* strains carrying DNA sequences modified in the region of the DNase I (EC 3.1.4.5)-hypersensitive sites −750 and −600 base pairs upstream from the *Sgs3* start site. Although both sites are developmentally specific, modifications in the −750 site have little or no effect on *Sgs3*-encoded transcript levels, whereas either deletion or replacement of sequences at the −600 site causes an important reduction in transcript levels. The element associated with the −600 site enhances *Sgs3* transcription when displaced with respect to the start site. This combined approach has defined sequence elements necessary both for normal transcript levels as well as the chromatin structure characteristic of *Sgs3* activity *in vivo*.**

The regulatory regions of eukaryotic genes are the site of a complex interaction between proteins and DNA sequences, as reflected in the specific chromatin structures that surround both active and inactive genes (1). Important advances stemmed from the fact that certain specific interactions can be reproduced on naked DNA *in vitro*, as well as from *in vivo* studies where plasmids are introduced at a high-copy number into cell lines. However, ensuring the physiological stoichiometry of the components in these experiments is difficult. This feat can be best accomplished by studies in transgenic animals, where a second DNA sequence is introduced into the germ line and experiences the same developmental accumulation of trans-acting factors as the normal resident chromosomal gene. In this way the DNA sequence is organized into a chromatin structure indistinguishable from that of the resident gene (2, 3). In view of the increasing evidence for multiple remote elements necessary for eukaryotic gene regulation *in vivo*, this approach may well be long, although ultimately it is subject to fewer artifacts than other approaches. In this report we use transgenic *Drosophila* to show that a developmentally specific remote DNase I (EC 3.1.4.5)-hypersensitive site (DHS) does, indeed, reflect an important regulatory element.

The salivary gland secretion protein gene *Sgs3* forms, with *Sgs7* and *Sgs8*, the 68C glue gene cluster (4). This gene is expressed, together with the other members of the glue gene family, only in salivary glands during the second half of the third larval instar. Its activity is repressed by the increase in ecdysteroid titer that also leads to secretion of the glue at pupariation (5). Extensive transformation analyses (6, 7) showed that regulation extends over 2.4 kilobases (kb) of 5′ flanking sequence, whereas some 300 base pairs (bp) of 3′ flanking sequence is sufficient for a normal developmental expression. The most remote region (−1.4 to −2.3 kb) contains quantitative elements necessary for abundant levels of *Sgs3*-encoded transcripts (7), whereas 130 bp of 5′ sequence are enough for low-level cell- and stage-specific transcription (6). Between these regions, analysis of the chromatin structure of the 68C fragment containing *Sgs3* and *Sgs7* (3) revealed a cluster of remote DHS sites at −470, −600, and −750 bp in larval glands. In prepupal glands the −600 site disappears while the −750 site is reinforced and becomes a clear doublet. A DNA fragment containing sequences associated with a similar cluster 5′ to *Sgs4* has been recently shown to direct expression of *Drosophila Adh* gene in salivary glands (8).

We have used oligonucleotide-directed mutagenesis to investigate the relationship between DNA sequences, chromatin structure, and gene expression *in vivo*. We show that sequences in the region of the −600 site are essential for formation of an active chromatin structure necessary for normal expression of *Sgs3*. The DNA fragment containing the DHS cluster enhances transcription both when inverted and displaced with respect to the proximal regulatory elements.

MATERIALS AND METHODS

Construction of Transposons. The nucleotide numbering used refers to the Oregon-R sequence (4), which applies equally to the Formosa sequence (9). All transposons are based on the Formosa genomic fragment of C20g71 (g71) (10). Modifications were made in the *Kpn* I (2108)–*Sal* I (4327) fragment [subcloned in pEMBL19 (11) to give pEMBL-(K-S)], and the derivatives were verified by DNA sequencing (12) and then were exchanged with the original *Kpn* I–*Sal* I fragment of C20g71 (see Fig. 1).

For deletions Δ20 (nucleotides 3842–3861), Δ63 (nucleotides 3827–3889), and Δ110 (nucleotides 3651–3761), single-stranded DNA from pEMBL(K-S) was hybridized with oligonucleotides complementary to nucleotides on either side of the sequence to be deleted and carrying a second *Bgl* II site in addition to the existing site (2189). Following the double primer approach (13) the extended gapped duplex was transformed into HB101 bacteria. For substitutions Δ20R, Δ63R, Δ110R, and Δ20R7/8, the newly created *Bgl* II sites in the pEMBL(K-S) derivatives were opened by partial digestion. To restore spacing of surrounding sequences, the fragments shown in Fig. 1 were ligated into the *Bgl* II site. The replacement fragments for Δ63R and Δ110R were the *Bam*HI–*Xba* I fragment from pA502 and the *Bam*HI–*Sst* I fragment from pA546, respectively. These fragments are neutral in enhancer-promoter spacing experiments with simian virus 40 (C. Schatz, personal communication). For pEMBL(K-S)Δ20R7/8 the oligonucleotides 5′-GATCCAATCTAAATTA-3′ and 5′-GATCTAATTTAGATTG-3′ were hybridized and ligated into the *Bgl* II site of pEMBL(K-S)Δ20. This restores the spacing, as in Δ20R, and inserts the

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Abbreviations: DHS, DNase I-hypersensitive site(s); g71, Formosa genomic fragment contained in C20g71.

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10-bp sequence (AATCTAAATT) found centered at −225 and −245 from *Sgs7* and *Sgs8*, respectively (3).

Displacement of the *Xba* I (3474)–*Bal* I (4072) fragment was by insertion of 105-, 598-, and 1053-bp fragments. The fragments *Bam*HI–*Sst* I from pA546, *Bam*HI–*Nru* I from pBR322, and *Bam*HI–*Ava* I from pBR322 were cloned into the *Bal* I site (4072). The deletion ΔB1.Pv removes the *Bal* I (4072)–*Pvu* I (4320) fragment. For inversion, the *Xba* I (3474)–*Bal* I (4072) fragment was filled in and recloned in the reverse orientation.

***P* Element Transformation.** Transformed lines were obtained in $ry^{506}C$-*S* (A. Spredling, Baltimore) as described (7, 10). A few lines containing double insertions detected in systematic Southern analyses are noted in Tables 1–3.

Analysis of Chromatin Structure. This analysis used hand-dissected salivary glands (approximately 200 per gel lane) as described (3).

RNA Extraction and Analysis. RNA extraction, gel electrophoresis, and transfer to nitrocellulose filters was as described (14). We have estimated expression of the Formosa *Sgs3* allele (800-base transcript, see refs. 9 and 14) relative to the resident allele (1100-base transcript) with two techniques: scanning of autoradiographs, as in previous studies, and scintillation counting of regions corresponding to bands cut from the nitrocellulose filters, as suggested by Vijay Raghavan *et al.* (6) (see Table 1 for examples). When expression of the inserted gene is abundant (>10% of that of the resident gene) measurements agree well. Below this level of expression, counting results in figures consistently higher than those obtained by scanning (see results from Δ63 and Δ63R). Background counts from the filter are estimated from RNA-free zones. When similar-sized pieces are counted below and above the band of *Sgs3*-encoded RNA in the *Drosophila* host strain $ry^{506}C$-*S* we can estimate degradation as well as the nonspecific smear above the band (neither of which are visible in short exposures). These two values are of the same order. Applying this technique to the construction g7 (10), estimated from scanning as <0.5%, we obtain 3% [which thus appears in accord with the 2–11% range of Vijay Raghavan *et al.* (6)] but obtain equally 3% for the zone *above* the Oregon allele. We conclude that when values are <5%, neither technique is totally satisfactory.

RESULTS

Modification of the DHS Region Sequence by Deletion or Replacement. In vivo *expression of genes bearing a modified −600 or −750 region.* A 10-bp tandem repeat is found at the −600 DHS (3), and both copies of this sequence were precisely deleted (Δ20) or replaced by a neutral DNA sequence (Δ20R). Equally, 63-bp deletion and replacement transposons (Δ63 and Δ63R) were constructed, again centered on the −600 site (Fig. 1). A second broader hypersensitive site at −750, which is particularly strong in cell types where *Sgs3* is inactive (3), was modified in the Δ110 and Δ110R constructions. Replacement constructs eliminate artifacts that may arise if an essential spacing between elements is disturbed by deletion.

Sgs3 expression was measured in transformed strains carrying these constructs (Table 1). In each case expression is limited to the third instar salivary gland (data not shown). Deletion of the 20-bp sequence results in expression that is between 10% and 20% of that of the resident gene, whereas its replacement leads to expression ranging from 25% to 40%. This suggests that at least two effects are superimposed in the

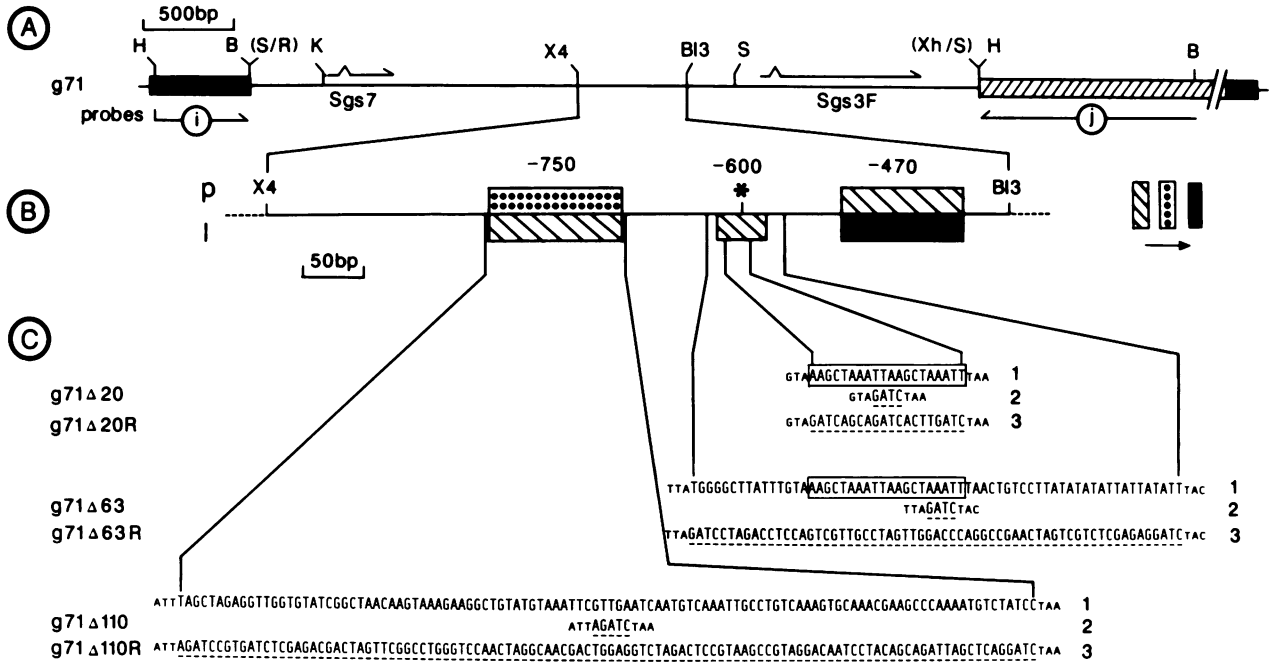

FIG. 1. Oligonucleotide-directed mutagenesis within the DNA fragment containing remote DHS of *Sgs3*. (*A*) A partial restriction map of the transposon g71 (10). Only the fragment contained within the *P* element extremities (solid box), inserted in transformants, is shown. The ry^+ marker gene is denoted by a broken hatched box. Restriction sites in parentheses are lost during cloning procedures. Above, arrows indicate the Formosa *Sgs7*- and *Sgs3*-transcribed regions with their introns. Below, arrows i and j denote the DNA probes used in DHS mapping (3). B, *Bam*HI; Bl, *Bal* I, H, *Hin*dIII, K, *Kpn* I, R, *Eco*RI, S, *Sal* I, X, *Xba* I, and Xh, *Xho* I. Where sites are followed by a number (e.g., X4), the fourth (*Xba* I) site in the 68C locus sequence is denoted (4). (*B*) A summary of DHS sites in the X4–B13 fragment in larval (l) and white prepupal (p) glands. Relative intensities of sites are shown in the order (weakest to strongest): hatched, dotted, and filled boxes. The asterisk denotes a site that disappears in prepupal glands. Numbers above give the distance from the center of the DHS to the *Sgs3* start site (from ref. 3). (*C*) Construction of modified g71 transposons. Three pairs of constructs are shown: Δ20/Δ20R, Δ63/Δ63R, and Δ110/Δ110R. In each case line 1 shows the wild-type sequence with nucleotides to be deleted in large capitals, line 2 shows the sequence resulting from the deletion, and line 3 shows the sequence following replacement. Unchanged flanking bases are shown by small capitals. Large capitals underlined denote nucleotides introduced either to create a *Bgl* II site (line 2) or to replace deleted sequences (line 3). The 10-bp tandem repeat at the −600 site is highlighted by a box.

Table 1. *Sgs3* expression in strains modified at the −600 and −750 DHS

C20 construct	Chromosomal location	Sgs3 expression, %†	
		Scanning	Counting
g71	g71-**1**(X), -2(3)	59, 65	65, 62
g71Δ20	g71Δ20-**1**(2)°*, -2(X), -3(3), -4(3), -5(2)*, -6(X)	15, 13, 18, 16, 16, 19	12, 17, 20, 17, 18, 20
g71Δ20R	g71Δ20R-1(2)*, -2(A), -**3**(3)	39, 32, 40	40, 29, 35
g71Δ63	g71Δ63-**1**(3), -2(X), -3(3)*, -4(3), -5(X)	4, 1, 4, 1, 1	7, 4, 6, 3, 3
g71Δ63R	g71Δ63R-1(X), -2(X), -3(3)*, -4(X), -**5**(3)	6, 2, 4, 1, 4	10, 5, 7, 3, 6
g71Δ110	g71Δ110-1(A), -2(X), -**3**(3)	68, 59, 63	71, 55, 55
g71Δ110R	g71Δ110R-1(2)°, -2(X)″, -3(2)*	66, 68, 64	70, 64, 66

°, Double insertion; *, hemizygous balanced lethal; A, autosome; ″, male lethal. Number following hyphen represents strain number; number within parentheses denotes *Drosophila* chromosome. Strains in bold type were chosen for chromatin studies. Hyphens are substituted for colons in g71 nomenclature to clarify series construction.
†See *Materials and Methods*. Percentage values are given as one dose of construct per haploid genome.

−600 region, one requiring a specific DNA sequence and the other requiring the physical separation of flanking elements. These flanking elements are disrupted by the deletion of the 63-bp sequence, which results in a low level of expression (Fig. 2 and Table 1). This effect appears sequence specific, because the replacement sequence does not increase transcript levels. Modifications of the −750 sequence have little or no effect on *Sgs3*-encoded transcript levels (Fig. 2).

Chromatin studies on the in vitro-*modified fragments*. The above results show that the DNA sequences modified at the −600 site are important for abundant expression of *Sgs3*. Because our working hypothesis is that this change results from their involvement of the hypersensitive site, we have undertaken a study in the DHS in strains carrying the modified upstream regions contained in Δ20, Δ20R, Δ63, and Δ63R, using the indirect end-labeling technique (15). Our mapping strategy uses the specific probes i and j (Fig. 3*A*); the results presented are those obtained with probe j.

The hypersensitive sites and changes between larval and prepupal glands outside of the fragment *Xba* I (3474)–*Bal* I (4072) are unchanged with respect to those found in g71 (Fig. 3) for these constructs. In the case of larval glands from both constructs Δ20 and Δ63 the narrow site at −600 (see g71) is absent, whereas those at −470 and −750 appear unchanged. This is seen as a clear "window" between the −470 and −750 DHS, absent in unmodified g71. Note that the Δ20-1 strain is hemizygous but carries two insertions (Table 1). For the replacements two distinct situations are seen. Replacement of sequences in Δ63R does not restore the −600 site. However, in Δ20R a hypersensitive site is present in larval glands (indicated by an arrow, Fig. 3*B*), and this site disappears in prepupal glands (see bottom of Fig. 3). In this last case the developmental changes of the −470 and −750 sites, associated with the cessation of *Sgs3* activity, are the same as in g71.

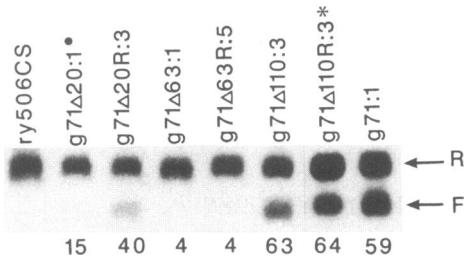

FIG. 2. *Sgs3* expression in transformed lines. Analysis of transcripts from the resident (R) and transformed Formosa (F) genes in third instar larvae. A transformant of each construct is shown as an example. The level of Formosa RNA is expressed as a percentage of the RNA from the resident gene (figure beneath blots) determined by densitometric scanning of a shorter exposure of this probed blot. Strains are homozygous with the exception of Δ20:1 (•), which is hemizygous but carries two independent insertions on the second chromosome, and Δ110R:3, which is hemizygous with a single insertion (see Table 1). The value for Δ110R:3 (*) has been multiplied by two for comparison with the other lines.

For Δ110, the site at −750 is absent (thus confirming its location), but that at −600 is present (data not shown), which is consistent with the abundant expression of the gene in this construct.

Effect of Displacing or Inverting the DHS-Associated Regulatory Element. To investigate whether there are constraints on the position of the −600 region with respect to *Sgs3*, we have displaced it in the constructs ΔB1.Pv, +100, +600, and +1050 (Fig. 4*A*). The −600 sequence is now centered at −350, −700, −1200, and −1650 with respect to the *Sgs3* start site. An analysis of these transformants (Table 2) reveals abundant *Sgs3* transcripts in all these configurations (Fig. 4 *B* and *C*). The distance of the element from *Sgs3* is not critical; expression is similar in the ΔB1.Pv, wild-type, +100, and +600 lines, whereas the most extreme displacement (+1050 bp) leads to an expression that is, at most, reduced by a factor of two.

Previous experiments have shown that *Sgs3* expression is normal in the g71I construct where the 2.6-kb *Eco*RI–*Sal* I 5′ flanking fragment is inverted with respect to *Sgs3* (10). We have investigated this property for the *Xba* I–*Bal* I fragment that contains the DHS cluster. In this construct the −600 sequence is inverted and at −760 with respect to the *Sgs3* start site. Expression in X.B1I lines is comparable to that in g71 (Fig. 4*C*, Table 3).

Exchange of Elements Between *Sgs* Genes. We have suggested previously that in the inversion construction g71I (see above section) an element containing the 10-bp sequence, AATCTAAATT found at −225 or −245 from *Sgs7* and *Sgs8*, respectively, substitutes for the *Sgs3* −600 element. This sequence is a 9/10 match for one of the 10-bp repeats at −600 deleted in Δ20 (3), and when inverted, an 8/10 match for an essential region of the immunoglobulin heavy chain enhancer [AATGCAAATT- (16)]. However, *Sgs3* expression in these lines (g71Δ20R7/8) is no higher than in the Δ20R lines (Tables 1 and 3). As this sequence was inserted centrally in Δ20, with 5 bp of stuffer sequence on either side so as to maintain spacing of flanking sequences, we cannot exclude that this is an unfortunate choice and that activity would increase if the sequence replaces precisely one or the other of the 10-bp repeats. In the present construct the level of activity reinforces the conclusion from Δ20R that the spacing of adjacent sequences is an important element in the −600 DHS.

DISCUSSION

We have used transgenic *Drosophila* as a functional assay of a regulatory element some 600 bp upstream of *Sgs3*. This element was first recognized as a consequence of its particular chromatin structure, a DHS, both in front of a wild-type *Sgs3* in its normal chromosomal position at 68C and in front of a transduced *Sgs3* gene showing normal developmental regula-

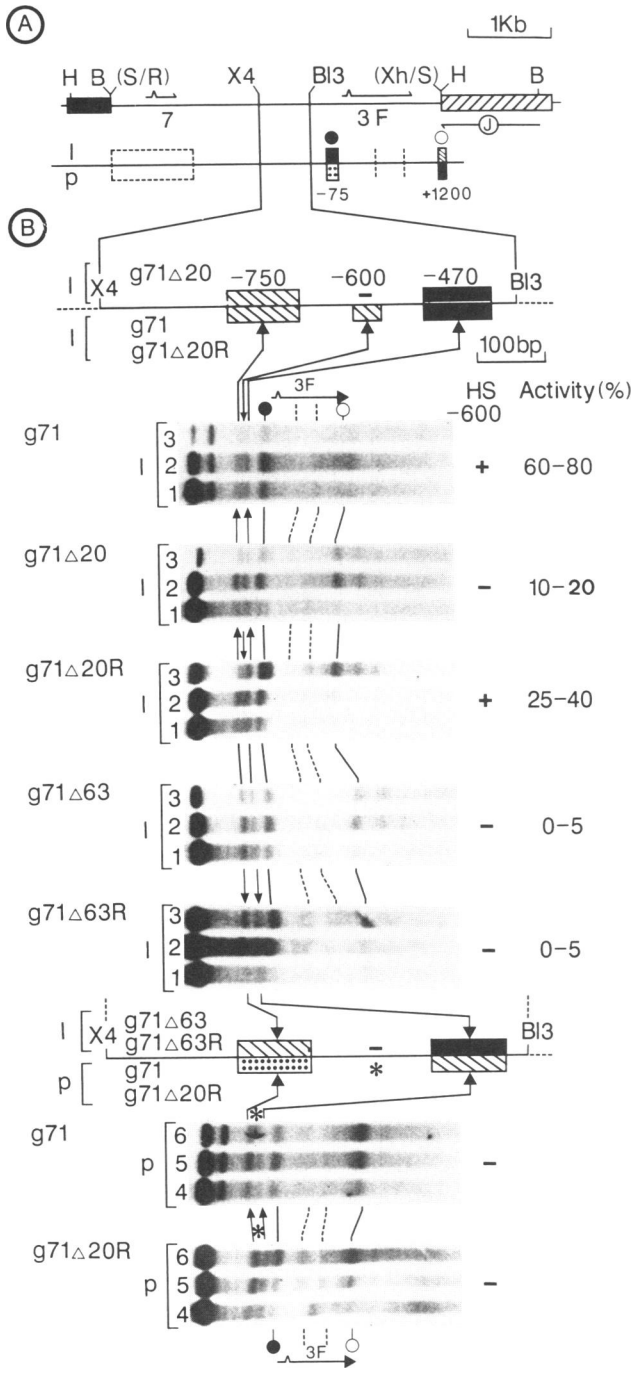

FIG. 3. Chromatin studies of transformants. (*A*) Summary of results obtained with g71 outside of the *Xba* I–*Bal* I region (ref. 3, see also Fig. 1). The DHS and their developmental changes, seen in all constructs, are shown as in Fig. 1*B*. The broken box in the region of *Sgs7* denotes a region of general DNase I sensitivity (3). Minor DHS within the *Sgs3*-transcribed region are denoted by broken lines. The major site at −75 and the site immediately 3′ to *Sgs3* are indicated by a full and open circle, respectively. (*B*) DNase I digestions (5′ at 20°C) of nuclei from homogenized salivary glands (see ref. 3 for full experimental details). Enzyme concentrations: lanes: 1, 3 μg/ml; 2, 6 μg/ml; 3, 8.5 μg/ml; 4, 3 μg/ml; 5, 8 μg/ml; and 6, 11 μg/ml. l, Larval glands; p, white prepupal glands. Strains showing the same pattern of DHS are grouped and shown above or below on one of the expanded summary diagrams. The −600 site (HS −600), characteristic of larval glands, is present (+) only in g71 and g71Δ20R. Its absence in all other strains is indicated by (−). It disappears in both strains in prepupal (p) glands as denoted by an asterisk (see also Fig. 1). Activity of a given construct refers to the range of values for different transformants. Lines used for chromatin analysis are those shown in Fig. 2. Note that g71 lanes contain a contaminating band close to the parental band (see ref. 3).

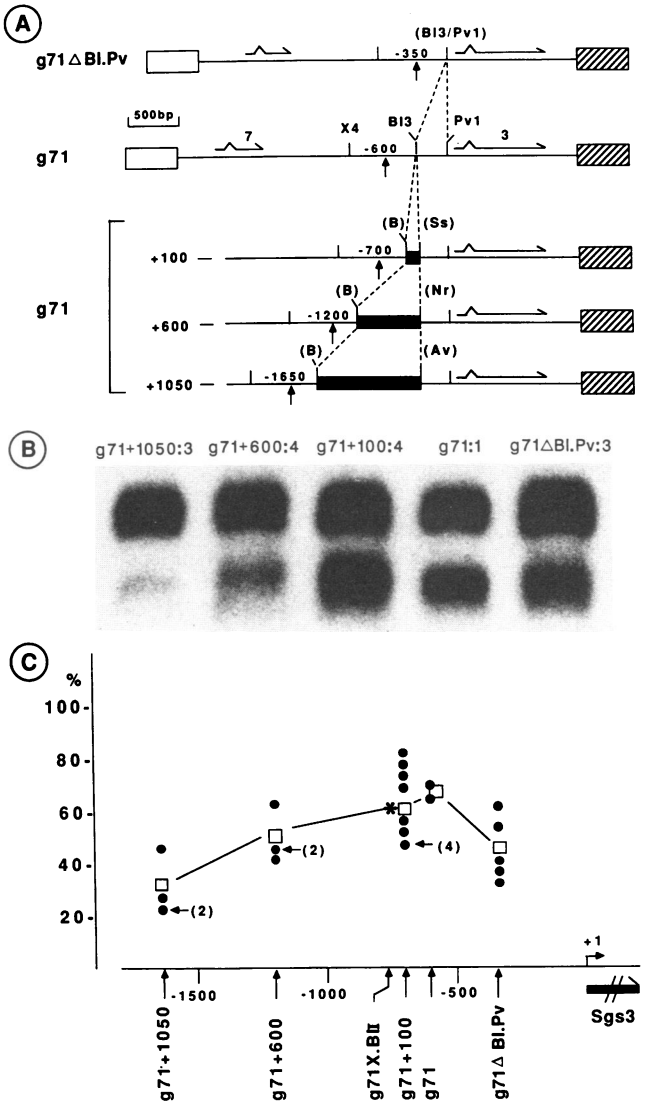

FIG. 4. Displacement of the DNA fragment associated with the −600 DHS 5′ to *Sgs3*. (*A*) The constructs ΔB1.Pv, +100, +600, and +1050 derived from g71. Av, *Ava* I, Nr, *Nru I*, Ss, *Sst* I. All other symbols as for Fig. 1. Restriction sites delineating the ends of the inserted fragments (solid boxes) are shown for orientation (see *Materials and Methods*). Arrows indicate the position of the −600 element with its distance from the *Sgs3* start site. (*B*) Examples of expression in transformed lines (see Table 2). (*C*) *Sgs3* expression as a function of the distance of the −600 element from the *Sgs3* start site. Mean value for each line (determined from at least three samples) is shown by a filled circle. Mean of these values is shown by an open square. When several lines give the same value, a number is shown in parentheses. g71 values are from the present set of experiments. An asterisk denotes the mean value of X.B1I lines (see Table 3).

tion (3). This analysis had suggested that a 10-bp tandem repeat might constitute an essential part of this element.

Table 2. Transformed strains in which the −600 DHS sequences are displaced

C20g71 construct	Chromosomal location of lines
ΔB1.Pv	g71ΔB1.Pv-1(X), -2(3)°*, -3(3), -4(2)*, -5(2)
+100	g71+100-1(3), -2(X), -3(3), -4(X), -5(3), -6(3)*, -7(3), -8(2)*, -9(2)°, -10(X)
+600	g71+600-1(2), -2(3), -3(2)*, -4(X)
+1050	g71+1050-1(2), -2(X), -3(3), -4(3)

All symbols as in Table 1.

Table 3. *Sgs3* expression in strains in which the −600 DHS sequences are inverted or substituted

C20g71 construct	Chromosomal location	Expression, %
X.B1I	g71X.B1I-1(2), -2(3), -3(X), -4(2), -5(3), -6(2)*, -7(2)	54, 92, 35, 47, 76, 61, 40
Δ20R7/8	g71Δ20R7/8-1(3), -2(2)*, -3(2), -4(3)*, -5(3), -6(X), -7(2), -8(2)	23, 35, 31, 25, 30, 23?, 25, 21

*, Hemizygous balanced lethal.-, :.

Although this sequence is important for *Sgs3* expression, it is only one factor necessary for both a normal DHS at −600 and high levels of *Sgs3* transcripts. This site should provide a model system for dissecting *in vivo* the structure of a DHS.

The Δ110 deletion apparently affects neither the expression of *Sgs3* nor the chromatin structure of the −600 DHS. A second large deletion (ΔB1.Pv) removes some 250 bp between the −600 region and the proximal promoter, without seriously reducing *Sgs3*-encoded transcript levels. These two large deletions, 5′ and 3′, respectively, to the −600 site, emphasize the importance of the sequences deleted in constructs Δ20 and Δ63.

This analysis has revealed something of the complexity of the −600 regulatory element, and we deduce that several factors are involved. Our working model is that a protein(s) binds to specific DNA sequences so as to create the structure that presents a DNA target sequence to a trans-acting regulatory protein and incorporates the finding that sequences adjacent to a DHS may be essential for its formation (17, 18). In this model the DNA target sequence is exposed to DNase I during our chromatin digests. Deletion of the 20-bp sequence may (*i*) remove a part of the exposed sequence including the target sequence; (*ii*) reduce the gap between two proteins bound to flanking sequences, thereby masking the recognition site for the trans-acting factor or introduce physical constraints if two normally separated DNA sequences bind to a single protein; (*iii*) remove a part of the binding site of the "fixed" protein(s); or (*iv*) be a combination of effects *i–iii*. When we replace this sequence by another 20-bp sequence, we reduce the physical constraints that increase accessibility of the site to the trans-acting protein. This is reflected by the appearance of a DHS in Δ20R. Using the present technique we are unable to distinguish this site from that found in g71. However, as expression is not fully restored, we probably do not restore either the wild-type chromatin structure or the target sequence. In contrast, the deletion of 63 bp destroys both the sequences that bind the protein(s) necessary to establish the site and the target sequence, and the replacement sequence cannot restore any aspect of the regulation.

The element localized within the remote DHS cluster is physically separable from the proximal promoter. The DNA sequences associated with the −600 site are able to enhance *Sgs3* transcription when displaced at least throughout the 1300-bp fragment defined by the ΔB1.Pv and +1050 constructs. McGinnis *et al.* (19) found that after the insertion of a 1.3-kb *hobo* element between the remote DHS of *Sgs4* and the proximal promoter, at −33 from the *Sgs4* start site, the chromatin structure was reconstituted on the displaced sequences. However, they found a dramatic reduction in *Sgs4* transcript levels—apparently a consequence of the induction of transcripts within the *hobo* element. That the DHS cluster follows the DNA sequence has been shown for *Sgs3* within the inversion construction g71I (see ref. 3).

Studies of the expression of chromosomal genes (as opposed to eukaryotic viruses) have shown that regulatory elements may extend over several kilobases (e.g., albumin, ref. 20; chicken lysozyme gene, ref. 21; mouse α-fetoprotein, ref. 22). It is clearly impossible to initiate a fine analysis, such as that used for viral enhancers and promoters (e.g., simian virus 40, ref. 23; polyoma virus, ref. 24; human cytomegalovirus, ref. 25), when such large regions are involved. We show here that chromatin studies constitute a powerful approach providing they can be linked to a functional assay. Somewhat unexpectedly, although the −750 DHS is prominent, particularly in prepupal glands, its deletion does not appear to alter *Sgs3* expression. In contrast, the results with the −600 site have already focused our studies to a relatively small essential sequence that is amenable to dissection at the single-base pair level.

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