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Sequences Involved in Temperature and Ecdysterone-Induced Transcription Are Located in Separate Regions of a *Drosophila melanogaster* Heat Shock Gene

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The transcriptional regulation of the *Drosophila melanogaster* hsp27 (also called hsp28) gene was studied by introducing altered genes into the germ line by P element-mediated transformation. DNA sequences upstream of the gene were defined with respect to their effect on steroid hormone-induced and heat-induced transcription. These two types of control were found to be separable; the sequences responsible for 80% of heat-induced expression were located more than 1.1 kilobases upstream of the RNA initiation site, while the sequences responsible for the majority of ecdysterone induction were positioned downstream of the site at −227 base pairs. We have determined the DNA sequence of the intergenic region separating hsp23 and hsp27 and have located putative heat shock and ecdysterone consensus sequences. Our results indicate that the heat shock promoter of the hsp27 gene is organized quite differently from that of hsp70.

The expression of eucaryotic genes is regulated in a temporal, spatial, and quantitative fashion during the development of an organism. Discernment of the mechanisms underlying these different levels of control is essential for the understanding of the molecular basis of differentiation and the maintenance of the differentiated state. An important step in defining the mechanisms of gene regulation is the elucidation of the DNA sequences that are responsible for mediating the transcriptional activity of genes.

Heat shock, the ubiquitous response of all organisms to a sublethal temperature elevation, has been studied extensively in both *Drosophila melanogaster* and *Escherichia coli* (see references 36 and 47 for a review) and constitutes an ideal model system in which to study the molecular basis of gene regulation in eucaryotes. In *D. melanogaster*, the response involves the abundant production of seven proteins, the heat shock proteins, within minutes of a temperature shift from the normal ambient growth temperature of 18 to 25°C to the elevated temperature level of 35 to 37°C. This response involves both the transcriptional activation of the genes encoding these proteins and the preferential translation of the corresponding mRNAs, while nearly all transcriptional and translational activity of non-heat shock genes is shut off. The molecular analysis of these genes has shown that they do not contain introns (with the exception of hsp83) and are clustered within five cytogenetic loci on chromosome 3. Although the biochemical function of the heat shock proteins remains obscure, it is known that they are vital for protection against heat and other potentially lethal physiological stresses. The recent advances in the understanding of the biochemistry of the bacterial heat shock genes, however, will undoubtedly shed light on the function of the eucaryotic heat shock genes as well (36).

Subsets of the *D. melanogaster* heat shock genes have been shown to be expressed at high levels during specific stages of development, in the absence of a heat shock (28, 55). hsp22, hsp23, hsp26, and hsp27 (also called hsp28) can be induced in tissue culture cells by the steroid molting hormone 20-OH ecdysterone (20) and in late-third-instar larvae-prepupae when the titers of this hormone are highest (8, 51). hsp26 and hsp27 have also been shown to be strongly expressed in the nurse cells of mature ovaries; the mRNAs for these proteins are transported into developing oocytes where they are stored until blastoderm formation (28, 59). All four of these developmentally regulated "small heat shock genes", along with a fifth developmentally regulated non-heat shock gene (R), are located within a 13-kilobase region on the left arm of chromosome 3 at 67B1 (10, 51). Workers in other laboratories have recently published transcriptional regulation studies of these developmentally regulated heat shock genes in both heterologous monkey cells (1) and *D. melanogaster* cells (34).

In the experiments described in this publication we have used germ line transformation to study the regulation of the *D. melanogaster* hsp27 gene which has been shown to be strongly transcriptionally induced during heat shock and at two specific stages of normal development. We previously described the construction of a size variant of the hsp27 gene, when a 207-base-pair (bp) deletion was made in the protein-coding region such that the resulting gene encoded both an mRNA and a protein that was distinguishable in size from the normal endogenous hsp27 (18). When a plasmid containing this gene with 2.1 kb of upstream DNA sequence was transformed into the *D. melanogaster* germ line, it was found to be regulated both transcriptionally and translationally in a temporal and quantitative manner nearly identical to that of the endogenous hsp27 gene. The smaller mRNA corresponding to the altered, transformed hsp27 gene also appeared as stable as the normal endogenous gene product, although RNA stability does not seem to be as important a regulatory factor as with hsp70 mRNA (49). These results indicated that hsp27 can be removed from the other similarly regulated genes in its cluster and still retain all of the expected transcriptional regulatory features. In the work described in this paper we used this variant gene (hsp18.5) to study the transcriptional regulation of the hsp27 gene during both heat shock and normal development. By deleting increasing amounts of the DNA sequences located upstream of this gene, we defined regions of DNA that have distinct roles in both types of regulation.

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MATERIALS AND METHODS

Maintenance of *D. melanogaster* stocks and germ line transformation. *D. melanogaster* stocks were grown at 21°C and 75% relative humidity. Injection of *D. melanogaster* embryos was carried out essentially as described by Rubin and Spradling (43). Preblastoderm-stage embryos were collected by placing a petri dish containing a mixture of agar and molasses on the mouths of half-pint plastic bottles containing 6-day-old ry^{506} flies (15) in which >75% of the genome had been crossed out with a wild-type Canton S stock to increase viability (T. Hazelrigg and G. Rubin, personal communication). The adults were allowed to lay eggs for 30 to 60 min. Embryos were dechorionated with 50% Clorox (2.6% sodium hypochlorite) (Clorox Co., Oakland, Calif.), attached to a cover slip with a small strip of nonlethal doublestick tape, covered with halocarbon oil, and injected with a supercoiled plasmid mixture of a Carnegie 20 construction (300 μg/ml) and either pπ25.1 (100 μg/ml) or pπ27wc (300 μg/ml) (22). The cover slips with the injected embryos were then moved to a petri dish containing moist filter paper, and the hatched larvae were transferred to standard food vials after 24 to 36 h and allowed to develop into adults. The adult flies were then back-crossed to the ry^{506} parental stock, with germ line transformed progeny of this cross selected by the wild-type eye color resulting from the expression of the xanthine dehydrogenase gene. All lines were either balanced or made homozygous, such that the allelic frequency of the transformed gene could be determined in each individual transformed line. Homozygous lethal and homozygous sterile insertions were balanced with *Cy0* if on chromosome 2 or with *TM3,ryRK* if on chromosome 3 (24; R. Karess and G. Rubin, personal communication). Although X chromosome insertions were observed, these were not included in this study, to avoid the complications of dosage compensation variables.

Isolation of nucleic acids, Northern and DNA sequence analysis, and in situ hybridization. Plasmid DNA was isolated by standard techniques (26). RNA was prepared by lysing the corresponding tissues in 4 M guanidine isothiocyanate–0.2% *N*-lauroyl sarcosine–150 mM mercaptoethanol–12.5 mM EDTA–50 mM Tris hydrochloride (pH 7.5) in a Dounce homogenizer. After the addition of an equal volume of 100 mM sodium acetate (pH 5.0) and two cycles of buffered phenol–chloroform–isoamyl alcohol–8-hydroxyquinoline (50:49:1:0.1) extraction at 65°C, followed by 10 min on ice, the RNA was precipitated with 1/10 volume of 3 M sodium acetate (pH 5.0) and 3 volumes of ethanol. The crude RNA pellet was suspended in water and then extracted with buffered phenol–8-hydroxyquinoline (100:0.1) at 65°C, followed by storage on ice as described above. The aqueous phase was extracted with chloroform and then precipitated with sodium acetate and ethanol as described above. RNA pellets were resuspended in water, a scanning optical density measurement was taken to determine purity and RNA concentration, and then the pellets were stored as an ethanol precipitate until use.

The DNA sequence of the hsp23-hsp27 intergenic region was determined through a combination of chemical degradation (30) and chain termination (45) methods. RNA samples were loaded on a 23-cm-long nonsubmerged 1% agarose–formaldehyde gel and blotted onto Biodyne transfer membranes (Pall Ultrafine Filtration Corp., Glen Cove, N.Y.) with 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Filters were baked at 80°C for 1 h and then washed for 30 min in 2× SSC–0.1% sodium dodecyl sulfate at 42°C to remove residual agarose. Prehybridization, hybridization, and washing of filters were done as specified by the manufacturer. Radioactive probes were either nick-translated (27) or random-primer-extended (13) hsp18.5 DNA or hsp27 DNA; a single high-specific-activity nucleotide precursor (>3,000 Ci/mmol; ICN Pharmaceuticals Inc., Irvine, Calif.) was used. At the hybridization stringencies used, preferential hybridization of either probe to either RNA was not observed.

In situ hybridizations were performed by dissecting late-third-instar larva salivary glands from either transformed homozygotes or nonbalanced heterozygotes in 45% acetic acid and squashing the polytene chromosomes as described by Pardue and Gall (37), except that the RNase and 2× SSC hydration steps were not performed. Hybridization and visualization of biotinylated DNA probes were carried out by using the reagents and instructions supplied with the Detek I-hrp system (Enzo Biochem, Inc., New York, N.Y.). hsp18.5 DNA was biotinylated by nick translation or random primer extension in the presence of biotin-11-dUTP (Bethesda Research Laboratories, Gaithersburg, Md.).

Plasmid constructions. p27P was constructed by introducing the 207-bp deletion contained in p27C (18) into p88.1 (10). The single *Pst*I site was then changed to a *Sal*I site as follows. p27P was digested with *Pst*I, and the 3′ cohesive termini were digested with T4 DNA polymerase (New England BioLabs, Beverly, Mass.) to create blunt ends (26). The DNA was phenol extracted, precipitated, and resuspended in TE (10 mM Tris [pH 8.0], 1 mM EDTA). One microgram was then added to 0.01 optical density units of kinased *Sal*I linkers (New England BioLabs) and ligated with 1 μl of T4 DNA ligase (Bethesda Research Laboratories) in 20 μl of LKB buffer (26) for 15 min at 16°C. The ligation reaction was then diluted to 100 μl of LKB and allowed to ligate for an additional 4 h to recircularize the plasmids. Fifty microliters was then used to directly transform frozen, competent *E. coli* HB101 cells. At least 50% of the Tet^r colonies obtained following this procedure contained between one and eight linkers in place of the *Pst*I site (sequence data not shown). The *Sal*I fragment of this plasmid (p27P-S) containing the hsp27 gene was inserted into the *Sal*I site of the transformation vector Carnegie 20 (44).

p27A was constructed by first ligating the *Eco*RI fragment of p27C to the *Eco*RI site of pUC8 (54, 58) such that the *Hin*dIII site of the polylinker was adjacent to the 5′ end of hsp23. This plasmid was then cut with *Sma*I and *Xho*I, and the vector fragment was ligated to a 370-bp *Aha*III-*Xho*I fragment of the same plasmid (designated p27A). The 1.3-kb *Sal*I fragment of this plasmid was then inserted into the *Sal*I site of Carnegie 20.

p27X was constructed by inserting the 1.2-kb *Xba*I fragment of p88.5 (10) into the *Xba*I site of p27P-S, in the orientation such that 1.1 kb of 5′ sequence was reconstructed. The hsp27 coding region was again cut out with *Sal*I and inserted into Carnegie 20.

RESULTS

Establishment of transformed lines. DNA fragments containing the altered hsp27 gene (hsp18.5) with various amounts of 5′ sequences were cloned in the *Sal*I site of the transformation vector Carnegie 20 (44) as described in Materials and Methods. Figure 1A shows a restriction map of the region surrounding the hsp27 gene and the size and location of the different fragments used in the transformation experiments. The orientation of the inserted fragment in the

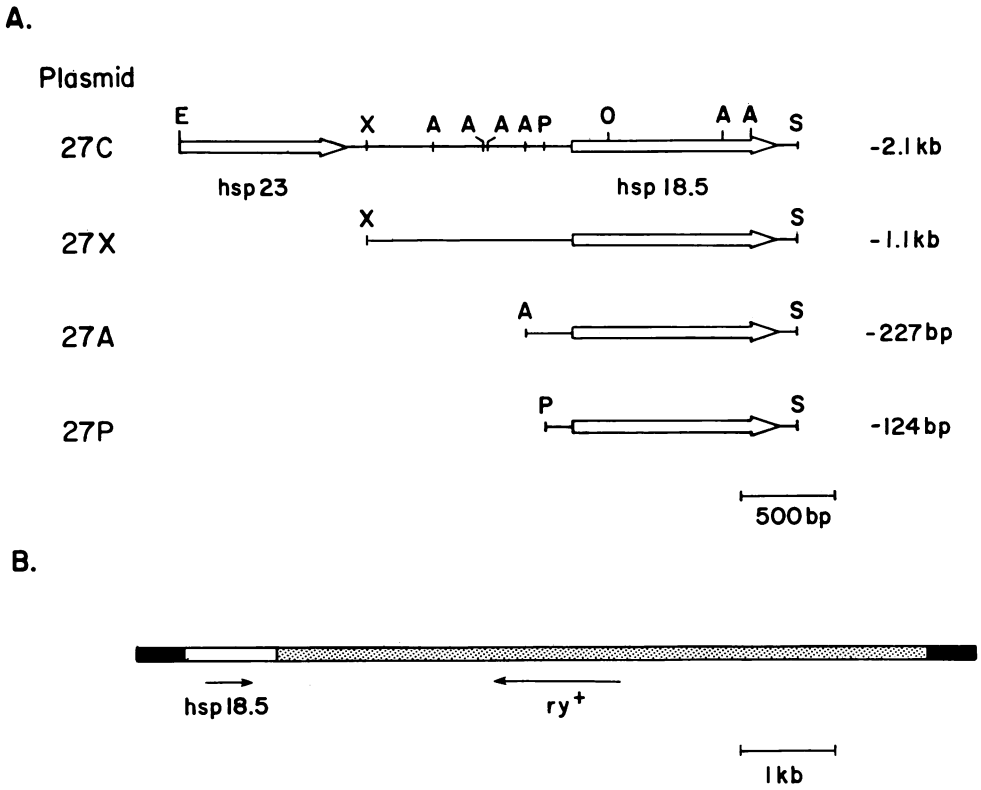

FIG. 1. Plasmids used for transformation. (A) DNA fragments containing the altered hsp27 gene (hsp18.5) that were inserted into the *SalI* site of Carnegie 20 and used for transformation. Although p28C includes most of the coding region of hsp23, it neither includes the TATAA homology for this gene nor the RNA initiation site and is probably not expressed. Abbreviations: A, *AhaIII*; P, *PstI*; X, *XbaI*; S, *SalI*; E, *EcoRI*; O, *XhoI*. (B) Organization of a transformed plasmid as it would appear inserted into the chromosome of the fly. Symbols: ■, insertion sequences of the P element transposon; hsp18.5, altered hsp27 gene; ry^+, wild-type gene for xanthine dehydrogenase which serves as the phenotypic transformation marker. The direction of transcription for each gene is shown by the arrows.

vector was such that the 3′ end of the hsp27 gene was adjacent to the 3′ end of the xanthine dehydrogenase (Xdh^+) gene, which was present as the germ line transformation phenotypic marker (Fig. 1B). The altered hsp27 and Xdh^+ genes were thus transcribed in opposite directions. This orientation was chosen so that any *cis*-acting effects of the xanthine dehydrogenase gene could be minimized. Recombinant Carnegie 20 plasmids containing the hsp18.5 gene were then injected into ry^{506} (Xdh^-) preblastoderm embryos by using either pπ25.1 (35) or pπ25.7wc (21) as a helper plasmid to provide the transposase activity in *trans*. Germ line transformants were selected and either balanced or made homozygous as explained in Materials and Methods, such that the allelic frequency of the transformed gene could be determined in each individual transformed line. The cytological localization of each of the transformed hsp18.5 genes was determined by in situ hybridization to polytene chromosomes by using biotinylated DNA probes. Table 1 summarizes the chromosomal insertion sites and associated phenotypes of each of the transformed lines. It is important to note that the 63BC insertion site of the 27C-A line is in the heat-shock-inducible puff region of hsp83 and the 27X-A line has its insertion in the ecdysterone-responsive gene locus of *Sgs-3*, *Sgs-7*, and *Sgs-8* at 68C.

Analysis of sequences required for heat shock-induced transcription. To identify the *cis*-acting regulatory elements of heat-induced transcription, we studied the effect that the sequential deletion of sequences upstream of hsp27 had on the transcription of the gene after heat shock. We analyzed transformed fly lines harboring constructions containing 1.1 kb (plasmid p27X), 227 bp(p27A), and 124 bp (p27P) of sequences 5′ to the transcription initiation site, in adition to a previously described construct containing 2.1 kb of 5′ sequence (18). Total RNA was isolated from adult flies kept at 21°C (control) and flies maintained at 36.5°C for 45 min (heat shock). The RNA was then electrophoresed on a 1% agarose–formaldehyde gel, transferred to a nylon membrane, and hybridized with ^{32}P-labeled hsp18.5 DNA. The results of this experiment are shown in Fig. 2. mRNA levels corresponding to the transformed hsp27 gene (hsp18.5) were calculated as a percentage of the endogenous hsp27 mRNA levels through integral scanning densitometry of the autoradiographs. The derived integrated percentages were then divided by the allelic frequency of the transformed gene in the individual transformed lines to arrive at the values displayed in Table 1. It should be noted that this calculation involves the assumption that there is no dosage compensation due to the different number of hsp27 homologous genes contained in the various transformed lines. Transformants containing 2.1 kb of 5′ DNA sequence have been extensively characterized elsewhere and were used in control lanes displaying the expected 0.95-kb mRNA corresponding to the transformed gene at levels equal to those of the 1.15-kb mRNA of the endogenous gene (14). Deletion of the sequences located between −2.1 and −1.1 kb has a profound effect on the accumulation of the hsp18.5 RNA after temperature elevation, decreasing its levels to 80% of those of the endogenous gene (Fig. 2). Deletion of the sequences located

TABLE 1. Summary of data for transformed lines

Line	Strain	Distance 5′ of gene	Chromosome	Position	Genotype[a]	Phenotype[a]	Allelic frequency	% transcription[b]	
								Heat shock	Ecdysone
28C	A	2.1 kb	3L	63BC	H	None	1.0	125	80
	B		2R	30A	B	Fem. Ster.	0.7	130	90
28X	A	1.1 kb	3L	68C	H	None	1.0	22	135
	B		3R	84D	B	Hom. Leth.	0.5	20	62
	C		2R	42F	H	None	1.0	21	
	D		3L	66A	B	Hom. Leth.	0.5		98
	E		3R	82C	H	None			
28A	A	227 bp	3R	95F	B	Fem. Ster.	0.7	16	64
	B		2+3L[c]	65D	B	Hom. Leth.	0.5	6	43
	C		3R	87AB	H	None	1.0	9	42
28P	A	124 bp	3L	61D	H	None	1.0	0	28
	B		3R	97B	H	None	1.0	0	23
	C		2L	39B	H	None	1.0	0	19
	D		3R	94B	H	None	1.0		44

[a] Abbreviations: H, homozygous; B, balanced; Fem. Ster., sterile female; Hom. Leth., homozygous lethal.

[b] Values are shown as a percentage of the endogenous (wild type) RNA accumulation, with 0% indicating values below the limits of detection (<3%). Values were determined by integral scanning densitometry of the autoradiographs shown in Fig. 2 and 3, as well as others not shown. Integrated values were averaged and then divided by the allelic frequency of the transformed gene in the fly line to arrive at the values displayed.

[c] This line contains a homozygous lethal insertion of unknown location on the second chromosome, and is also heterozygous for an insertion on chromosome 3.

between −1.1 kb and −227 bp causes a further slight decrease in the RNA accumulation after heat shock. Deletion of the sequences located between −227 and −124 bp lowers mRNA levels below the limits of our detection (<3%), apparently abolishing heat-inducible transcription. No higher-than-normal constitutive expression of the transformed gene was ever observed under non-heat shock conditions in adult flies, indicating that the hsp27 gene is probably under positive regulatory control during heat shock.

Effect of 5′ deletions on the developmental expression of the hsp27 gene. We previously showed that a hsp18.5 gene containing 2.1 kb of 5′ DNA sequence maintained a completely wild-type pattern of developmentally induced expression. High levels of RNA accumulation were found in both late-third-instar larvae-prepupae and early embryos (18). To study the role of different sequences located in the 5′ region of this gene on the temporal and quantitative regulation of transcription, we analyzed the developmental expression of the transformed hsp27 gene (hsp18.5) in the lines described above. Total RNA from at least six distinct developmental stages of *D. melanogaster* were analyzed by Northern blots for each line. A representative example of these experiments, with a fly line harboring the p27X plasmid containing 1.1 kb of upstream DNA sequence, is shown in Fig. 3. The temporal expression of the transformed gene was found to be identical to that of the endogenous one in this and every transformed line. RNAs corresponding to both the endogenous and transformed genes were present in 0- to 18-h-old embryos and early pupae; they were present at a low level in adults. Overexposure of the developmental Northern blots showed a very low basal level of expression in first-, second-, and early-third-instar larvae, which is not evident in Fig. 3. The basal expression level of the hsp18.5 gene was always very similar to that of the endogenous gene, independent of the construction used, probably representing unenhanced promotion from the TATAA box (32), which was not deleted in any of our constructs. Although the deletion of

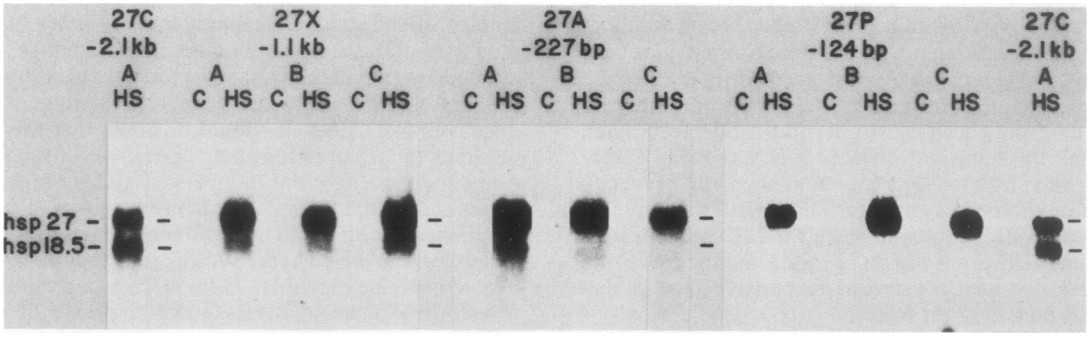

FIG. 2. Heat shock transcriptional analysis of transformed lines. Total RNA (10 μg) isolated from adult flies maintained at 25°C (C) and 36.5°C for 45 min (HS) was fractionated on an agarose-formaldehyde gel, blotted onto nylon filters, and hybridized to ^{32}P-labeled hsp18.5 DNA. The individual fly lines transformed with the indicated plasmid containing the indicated amounts of DNA sequences upstream of the transcription initiation site are shown. The relative transcriptional activity values, which take into account the allelic frequency of the transformed gene in each fly line, are shown in Table 1.

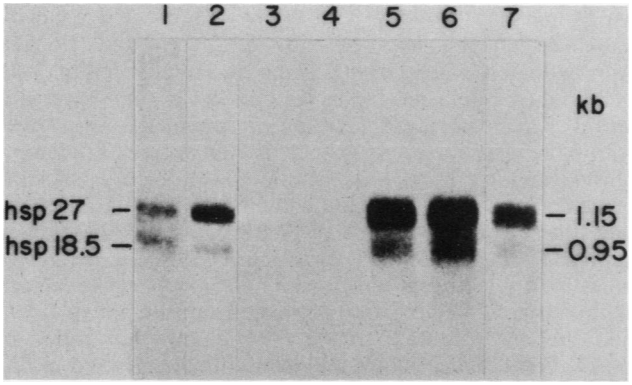

FIG. 3. Temporal expression of the endogenous versus transformed genes during development. Total RNA (15 μg) isolated from different developmental stages of the 28X-D transformed line harboring an hsp18.5 gene containing 1.1 kb of upstream DNA sequence was fractionated on an agarose-formaldehyde gel, blotted onto a nylon membrane, and hybridized with ^{32}P-labeled hsp18.5 DNA. Lane 1 contains 1 μg of p28C-A heat shock RNA as a marker. Developmental stages are as follows: lane 2, 0- to 18-h-old embryos; lane 3, first-instar larvae; lane 4, second-instar larvae; lane 5, late-third-instar larvae; lane 6, prepupae; lane 7, 2-day-old pupae.

sequences upstream of the hsp27 gene did not appear to have any effect on the normal temporal pattern of expression, it did have a significant effect on the quantitative expression of the transformed gene. The hsp27 homologous RNAs accumulated during the prepupal stage, when hsp27 is under the maximal influence of the steroid hormone ecdysterone, are shown for each transformed line in Fig. 4. The quantitation of the relative levels of RNA on these and similar Northern blots is summarized in Table 1. Although some position effect was observed, the transformed lines harboring an hsp27 gene containing 1.1 kb of 5′ sequence accumulated approximately equal amounts of RNA corresponding to the endogenous and transformed genes. Transformants containing 227 bp upstream of the transformed gene accumulate hsp18.5 RNA at levels that are approximately 50% of those of the wild type, while deletion of the sequences located between −227 and −124 bp causes a further decrease in the transcription of the transformed gene to levels that are approximately 30% of those of the endogenous gene.

The hsp18.5 RNA accumulation during normal development seems in general to be much more sensitive to chromosomal position than does the heat-induced accumulation (Table 1). Particularly interesting is the 27X-A transformed line, which accumulates hsp18.5 RNA at 135% of the levels of the endogenous gene. The transformed p27X plasmid in this line has inserted into the developmentally puffed 68C locus, in close proximity with three of the ecdysterone-induced glue genes, *Sgs-3*, *Sgs-7*, and *Sgs-8* (14, 17). It is possible that the higher transcriptional levels observed for this transformant are due to a transcriptional enhancement of the transformed gene, as a result of its proximity to the similarly regulated glue genes.

The RNA corresponding to the hsp27 gene has also been found in high concentrations in early embryos (0 to 18 h old) (28), where it accumulates owing to its transcription in ovarian nurse cells and subsequent transport to the developing oocytes (59). We expected the ovarian transcriptional activity of the deleted transformed genes to parallel the ecdysterone-induced activity in prepupae as a result of the steroid-hormonal influence on oogenesis, which has been best studied in the mosquito (3). We found this indeed to be the case. As shown for the 27X-D line in Fig. 3, the amount of transcript present in embryos very nearly parallels the levels found in prepupae. Approximately 30% of ovarian expression is still present, with only 124 bp of upstream sequences (data not shown). We also studied the RNA accumulation in ovaries and carcasses of the 27P-A line, which harbors an hsp18.5 gene containing only 124 bp of upstream sequence. High levels of hsp18.5 RNA were found in the ovaries, while RNA levels were undetectable in the carcasses, indicating correct tissue specificity in addition to the correct temporal regulation (data not shown).

Heat shock induction of the transformed gene in prepupae. The results described above indicate that the heat shock response is profoundly affected by deletion of sequences far (>1.1 kb) upstream of the hsp27 gene, while the prepupal and ovarian controls are located within sequences closer to the gene. It is thought that factors that influence transcription at great distances from the transcriptional start points

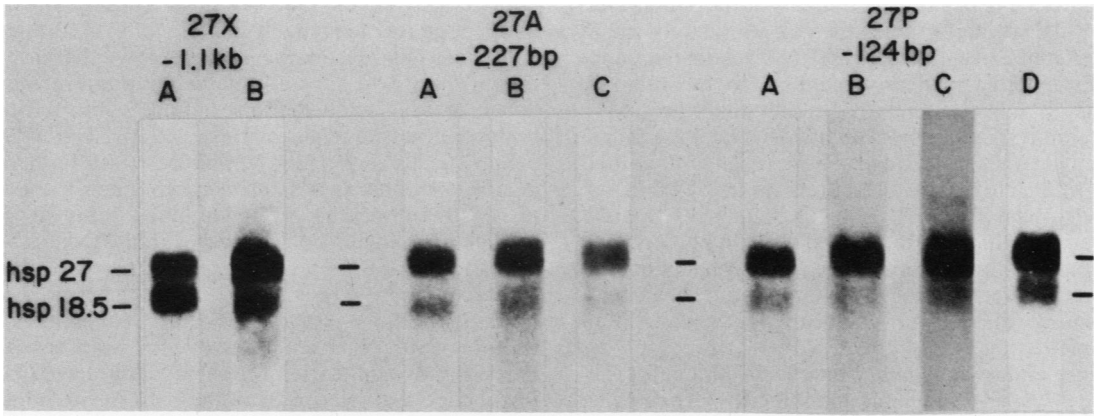

FIG. 4. Steroid hormone-induced transcriptional analysis of transformed lines. Total RNA (15 μg) isolated from prepupae of each transformed line was fractionated on an agarose-formaldehyde gel, blotted onto nylon membranes, and hybridized with ^{32}P-labeled hsp18.5 DNA. The plasmid used for transformation, the amount of DNA sequence 5′ to the transcription initiation site of the transformed hsp18.5 gene, and the fly line used are shown above each lane. hsp27 refers to the 1.15-kb RNA product of the endogenous gene, and hsp18.5 refers to the 0.95-kb RNA product of the transformed gene. The relative transcriptional activities, which take into account the allelic frequency of the transformed gene in each fly line, are shown in Table 1.

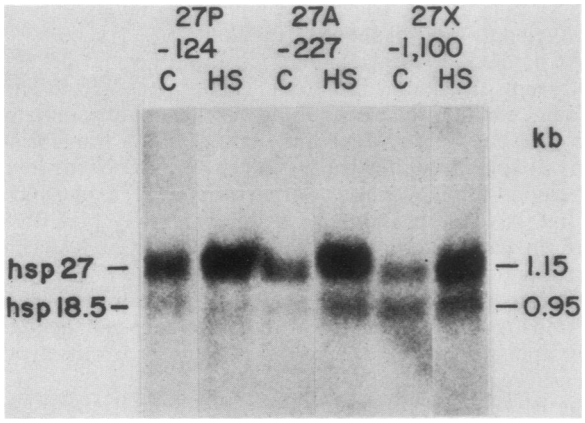

FIG. 5. Heat shock-induced expression in prepupae. Total RNA (10 μg) isolated from prepupae maintained at 25°C (C) or heat shocked for 45 min at 36.5°C (HS) was fractionated on an agarose-formaldehyde gel, blotted on a nylon filter, and hybridized with ^{32}P-labeled hsp18.5 DNA. The plasmid used for transformation and the amount of DNA sequence 5′ to the transformed hsp18.5 gene are shown above each lane. The RNA species corresponding to the endogenous wild-type gene is designated hsp27, while the RNA produced by the transformed gene is designated hsp18.5.

probably do so by altering the regional chromatin structure, making the promoter more accessible to other transcription factors that have a more direct influence on transcriptional promotion (16). We reasoned that if the transformed hsp27 gene is being actively transcribed owing to steroid hormone control, it might have an "open" chromatin structure that could restore some of the 80% of heat shock control that is lost upstream of −1.1 kb. To test this hypothesis, RNA was prepared from control (21°C) and heat-shocked (36.5°C) prepupae from transformed lines 27P-A, 27A-A, and 27X-A (Table 1). The Northern analysis of these RNAs is shown in Fig. 5. It can be seen from the control lanes of each line that the transformed gene is expressed as a result of the influence of ecdysterone at the same quantitative levels as derived from the developmental Northern blot experiments described above. After a heat shock, the 1.15-kb band corresponding to the endogenous gene product increases in intensity by about a factor of 4, while the 0.95-kb RNA band corresponding to the hsp18.5 gene remains at the same level as the control (27P-A line) or increases by a relatively small amount (27X-A and 27A-A lines). The RNA accumulation in heat-shocked prepupae is nearly identical to the relative accumulations found in adults, indicating that the ecdysterone induction is unable to rescue the disabled long-range control of the heat shock response. These results imply that if sequence-specific chromatin alterations are involved in the 80% decrease of heat shock RNA when sequences located 1.1 kb upstream of hsp27 are deleted, the hypothetically open chromatin structure present during the strong induction by ecdysterone is different enough from the sequence dependent heat shock chromatin structure to make them unexchangeable.

DNA sequence of the hsp23-hsp27 intergenic region. To see whether there were any sequence homologies between the regions we had defined as functionally important and the known heat shock transcription factor (HSTF) consensus binding site (2), we determined the DNA sequence of hsp23-hsp27 intergenic from the clone used in these studies, which was isolated from a Canton S *D. melanogaster* stock (10) (Fig. 6). The Oregon R sequence of the coding regions of hsp23 and hsp27, along with approximately 250 bp of 5′ flanking sequence, has been previously reported (19, 52). Approximately 550 bp of the 1.4 kb presented overlaps with that already published for hsp23 and hsp27. Our sequence varies significantly (6%) from the published sequences within the overlapping regions. This high degree of polymorphism is predominantly due to additions or deletions of small blocks of repetitive DNA and could be representative of the time that these two strains of *D. melanogaster* have been kept as isolated laboratory stocks, or could be indicative of the lack of selective pressure on a noncoding sequence such as this. Figure 6 shows the sequence from the polyadenylation site of hsp23 (52) to the protein initiation codon of hsp27. The breakpoints of the deletion mutants used in this study are shown. Sequence homology searches (21; BIONET SEQ/SEARCH/HOM) between the heat shock consensus sequence (41) and the 2.1-kb region upstream of hsp27 determined to be necessary for full heat shock expression elucidated the four sequences denoted by asterisks. These four sequences are also displayed in Fig. 7A. Since at least one published result concerning the heat inducibility of a transformed hsp70 was explained by the fortuitous inclusion of a heat shock consensus sequence in the adjacent vector sequences (9, 50), we checked the neighboring sequences of our plasmid constructions for such homologies, and none was found. Also shown are the large blocks of alternating purines and pyrimidines which are in common to nuclear scaffold attachment sites neighboring the *D. melanogaster* hsp70 genes (29, 33).

DISCUSSION

Heat-inducible promoter of hsp27. The transcriptional regulation of the *D. melanogaster* heat shock genes has been intensively studied in a number of laboratories. The organization of the promoter of the major *D. melanogaster* heat-induced polypeptide gene hsp70 is now well understood (see reference 2 for a review). A 55-bp region of the hsp70 gene located between −40 and −95 bp upstream of the RNA cap site has been found to be necessary for full levels of heat-induced transcription through germ line transformation experiments conducted by three independent groups of investigators (9, 12, 50). This same region has been found to contain two HSTF binding sites that are protected from digestion by DNaseI in vitro and by *Exo*III in isolated nuclei after a heat shock (38, 57). The binding sites of HSTF correspond to sequences homologous to the heat shock consensus (40, 41), and it has been shown that the deletion of one of these two sites correlates with a severe drop in the levels of heat-induced transcription, while the deletion of both of these sequences abolishes transcription (9, 12, 50).

Statistically significant homologies (8/10-bp match) to the heat shock consensus sequence have been found within 150 bp upstream of the RNA initiation sites of all the *D. melanogaster* heat shock genes, with the exception of hsp27 (40). In fact, HSTFs have been found to bind regions of DNA containing these sites in all of the heat shock genes, again with the exception of hsp27 (C. Parker, personal communication). In addition, in vitro transcription experiments involving the use of purified HSTF have to date failed to produce a transcript from the hsp27 gene alone (Parker, personal communication). Two studies investigating the heat-induced transcription of the small heat shock genes transfected into monkey COS cells showed that hsp27 was either noninducible (1) or only nominally inducible (42) by heat shock, while hsp70, hsp22, and hsp26 were strongly induced, allowing their promoters to be mapped in this

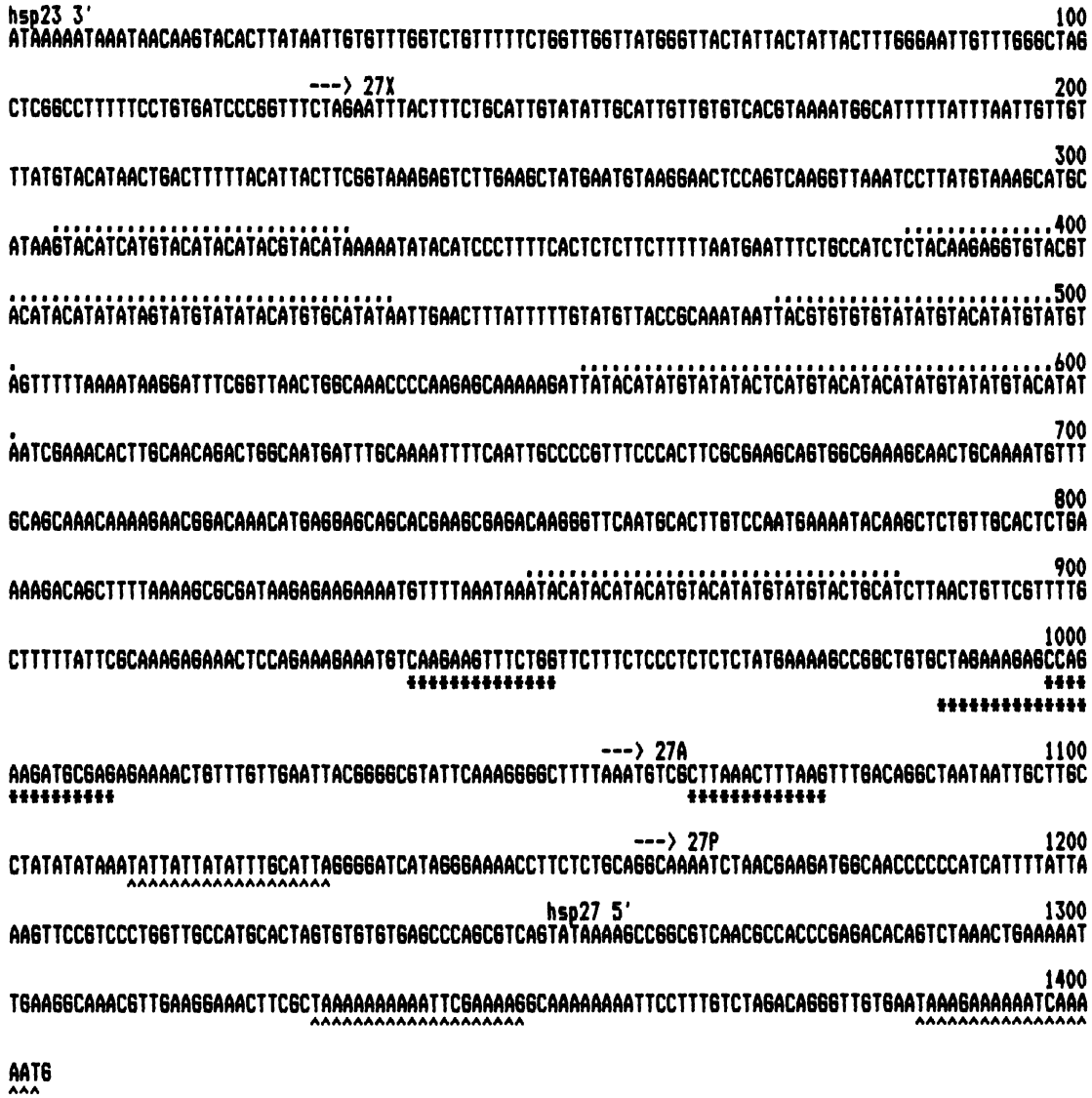

FIG. 6. Nucleotide sequence of the hsp23-hsp27 intergenic region. The DNA sequence of the hsp23-hsp27 intergenic region originating from a Canton S strain of *D. melanogaster* (10) was determined by a combination of chemical degradation (30) and chain-terminating (45) techniques. Both strands were sequenced. The presented sequence begins with the polyadenylation signal of hsp23 and ends with the protein initiation codon of hsp27 (52). Homologies to HSTF binding sites (see reference 2) are indicated by asterisks and are shown in Fig. 7A. Blocks of alternating purines and pyrimidines similar to those found in the nuclear scaffold attachment sites neighboring the hsp70 genes (29, 33) are indicated by dots above the sequence. p27X, p27A, and p27P indicate the precise breakpoints of three of the four deletion mutants used in this study. Homologies to functionally important regions of the *D. melanogaster* glue genes, shown in Fig. 7C, are indicated by ∧.

heterologous system (1). These studies, taken together, suggest that hsp27 possesses a heat shock promoter that is organized in a structurally or functionally different way from that of the well-characterized hsp70 genes. Our results indeed confirm this conclusion. We find that the sequences responsible for more than 80% of the heat-inducible transcription of this gene lie more than 1.1 kb upstream of the coding region, probably within the neighboring hsp23 coding region.

To see whether there were any HSTF binding site consensus sequences upstream of hsp27, we determined the DNA sequence of the intergenic region between hsp23 and hsp27 and then conducted computer homology searches between the consensus sequence and the 2.1 kb 5′ of hsp27 that we had determined was functionally important for full levels of expression. This search elucidated four 8/10-bp matches clustered within a 125-bp region approximately 300 bp upstream of the RNA initiation site, which are displayed in Fig. 6 and 7B. Our results indicate that these consensus-binding sites could, at best, be capable of directing 20% of heat-induced transcription. The inclusion of all four of these sequences, along with more than 500 bp of additional DNA sequence 5′ (27X plasmid), is still heat inducible but at a relatively low level. In contrast to hsp70, then, the DNA sequences responsible for 80% of heat-inducible transcription seem to be unrelated to the heat shock consensus sequence. If these consensus sequences are indeed important for the regulation of hsp27, the deletion of three of the four consensus sequences seem to have only a minimal effect on transcription, lowering levels from 20% of normal (27X

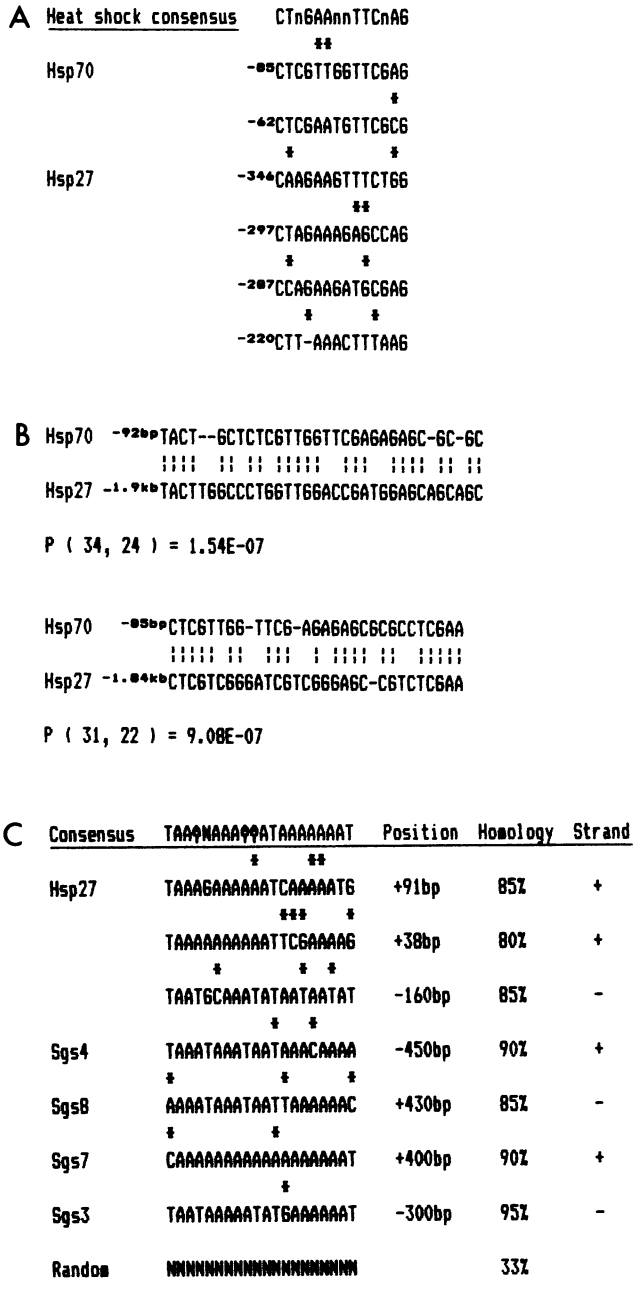

FIG. 7. Sequence homologies near the hsp27 gene. (A) Heat shock consensus sequence (40), with the two previously defined hsp70 homologs (50) and the hsp27 homologs discussed in this paper. Nonhomologies are indicated by an asterisk. (B) Two regions upstream of hsp27 that are homologous to a 40-bp region of the hsp70 promoter responsible for enhancing transcription. The −68- to −97-bp region of the hsp70 promoter, which has been shown to significantly enhance heat shock transcription (9, 12, 50), was compared with the −1.1- to −2.1-kb region of hsp27 gene by using the Bionet SEQ/SEARCH/HOM computer program. Two sequences located approximately 1.9 kb upstream of the hsp27 transcription initiation site, separated by 30 bp, are shown; their homologies to the hsp70 promoter are indicated by dashed lines. The probability of such a match occurring in random sequence is shown below each homology (P). (C) SEQH computer program-located homologies (21) beween regions of hsp27 that we have defined as functionally important and regions of the *D. melanogaster* glue genes that other studies have shown to be functionally important for ecdysterone-induced expression of these genes (4, 31, 35). Positions are relative to the transcriptional start sites, with + designating the noncoding (RNA) strand and − designating the coding (sense) strand. Sequences for the glue genes were obtained from references 14 and 35.

lines) to 6 to 16% of normal (27A lines). The variation observed with the 27A lines could simply be due to the increased influence of chromosomal location owing to the loss of buffering DNA (53), or could be due to a real loss of important sequences. The deletion of the last consensus sequence, however, correlates with an apparent loss of all heat inducibility (27P lines). It should be noted that although this last consensus sequence possesses perfect dyad symmetry, it is centered on a 1-nucleotide spacer instead of a 2-nucleotide spacer, as are all other functional consensus sequences. We conclude, then, that although putative HSTF binding site consensus sequences could be involved in the heat-induced regulation of hsp27, sequences located between −2.1 and −1.1 kb upstream of hsp27 are responsible for a fivefold enhancement of transcription and do not contain such consensus sequences.

We then conducted computer homology searches between the entire 55-bp hsp70 promoter region (−40 to −95 bp) and the −1.1 to −2.1-kb region we had defined as directing 80% of heat-induced hsp27 transcription. This search elucidated the two extensive homologies shown in Fig. 7B, which are located 30 bp apart in the hsp23 coding region. We also found large blocks of alternating purines an pyrimidines in the hsp23-hsp27 spacer similar to those found within nuclear scaffold attachment sites (33). These observations, taken together, make it tempting to propose a "circean scaffold" model for the heat induction of hsp27, such as that generally proposed by Mikovitch et al. (33). hsp70 promoter sequence homologies within the hsp23 coding region could serve to concentrate HSTF molecules near the "weak" consensus sequence binding sites downstream, thereby enhancing transcription from the otherwise weak promoter of hsp27. These two regions might be brought into close physical proximity through a nuclear scaffold attachment site such as those shown to neighbor the hsp70 genes (33). This model is pure speculation at best, although experiments are under way to test it.

Steroid hormone promoter of hsp27. Sequences responsible for the induction of transcription through the binding of steroid hormone-receptor complexes have been extensively studied in other systems and have been shown to behave as enhancer elements in the sense that they act in an orientation- and somewhat distance-independent manner and are often present in multiple copies around the promoter of these genes (7, 39, 46). Our data suggest that there are at least three regions of DNA upstream of the hsp27 gene needed for full developmental expression in response to ecdysterone; approximately 50% of ecdysterone-induced activity was lost between −1.1 kb and −227 bp, and sequences downstream of −124 bp were responsible for the remaining 30%. These results clearly indicate that the developmental control of this gene requires different sequences than those required for the heat shock induction. While RNA levels in prepupae are at approximately 100% of normal levels with 1.1 kb of upstream sequence, this same construction is capable of directing only 20% of heat shock transcription. Similarly, a construction containing 124 bp of upstream sequence is still capable of directing approximately 30% of ecdysterone-induced transcription, while this same construct is apparently incapable of responding to heat shock.

A model for the mechanism of the developmental induction of the small heat shock genes was recently proposed by Bienz (2). It was suggested that developmentally specific factors might modulate inactive HSTFs such that they could then bind the heat shock consensus-binding sites upstream of the small heat shock genes in the absence of a heat shock (2). Our results argue against this model, since sequences between −2.1 and −1.1 kb appear nonessential for developmental control, yet are required for the majority of heat shock control. Furthermore, the deletion to −124 bp, which deletes all of the putative heat shock consensus sequences and all apparent heat shock induction, is still capable of directing 30% of developmental transcription. Our results imply that the heat shock and developmental controls are separate and distinct.

Although the results presented here are the most complete analysis of sequences responsible for ecdysterone induction regarding any of the small heat shock genes, studies regarding some of the *D. melanogaster* glue genes have been published. These genes appear to be responsive to low titers of ecdysterone in third-instar larval salivary glands (17). A 95-bp region 450 bp upstream of the Sgs4 gene has been shown to be indispensable for its in vivo expression (31, 35). Also, P-element-transformation transcription studies of the *Sgs-3* gene have shown that sequences around the neighboring *Sgs-7* gene, more than 1.0 kb upstream of *Sgs-3*, are essential for wild-type expression (4). Using the regions of DNA that we had determined to be functionally important for ecdysterone-induced expression of hsp27, we conducted homology searches to the functionally important regions of the glue genes. We found a ≥ 20-bp region that was extremely dA rich common to all of these regions and which is displayed in Fig. 7C. Although poly(dA) stretches are relatively common in *D. melanogaster* DNA, the length of the homology with its very low likelihood of appearing in random sequence makes its presence in all of these functionally defined regions significant.

Correlation of nuclease-sensitive sites and deletion results. DNA sequences sensitive to certain nucleases have been observed to surround the transcription initiation sites of a number of genes in both native chromatin and supercoiled plasmids harboring these genes. In some cases, the presence of these sites has been correlated with the transcriptional activity of these genes (5, 6). In *D. melanogaster*, it has been shown that the transcriptional activity of the *Sgs-4* glue gene (which is expressed solely in the salivary glands of late-third-instar larvae) is related to the presence of temporally and spatially specific DNase I hypersensitive sites upstream of the gene (31, 35). One of these sites, positioned 450 bp upstream of *Sgs-4*, is contained within a 95-bp region which, when deleted, abolishes the expression of this gene (31, 35).

The nuclease-sensitive regions of the *D. melanogaster* heat shock genes have been extensively mapped in both native chromatin isolated from embryos (11, 23, 56) and supercoiled plasmids harboring the heat shock genes (25, 48). Surprisingly, the DNA sequences in the hsp70 gene corresponding to both the DNaseI-hypersensitive sites present in chromatin and the S1 nuclease-hypersensitive sites present in supercoiled plasmids have been shown to be unnecessary for full levels of heat-induced transcription (9, 12, 50). The major DNaseI-hypersensitive sites of hsp27 found in native chromatin are located at −18 and −82, with minor sites at −293 and −552 (11). None of these sites lies within regions which contain critical functions for heat shock induction in the one-dimensional experiments presented here. These sites could, however, have a functional role which is not detected in our series of deletions. A clearer correlation could be drawn between the sequences that are important for steroid hormone induction and the DNase-hypersensitive sites, in that the region harboring the two major DNase-hypersensitive sites (−18 and −82) is needed for 30% of transcriptional activity, while the region containing the two remaining weaker sites (−293 and −552) is required for an additional 50% of activity.

It is important to note that for the ecdysterone-inducible glue gene Sgs-4, the hypersensitive sites that were correlated with the expression of the gene can only be identified upon transcriptional induction in a tissue- and stage-specific manner. However, the hypersensitive sites of hsp27 and the other heat shock genes have only been studied in chromatin extracted from embryos, in which the hsp27 gene is not developmentally induced. It is probable, then, that a new set of hypersensitive sites appear upstream of hsp27 upon its transcriptional activation in prepupae and that these correspond to the sequences we determined as being necessary for expression. Preliminary results from our laboratory have indicated that this is indeed the case: an array of hypersensitive sites appear in prepupae which correspond to the regions which we have defined as functionally important for ecdysterone-mediated expression. Furthermore, these sites lie very near the poly(dA) tracts shown in Fig. 7C. Since the poly(dA) tract shown for the Sgs-4 gene also correlates with a hypersensitive site in a region indispensable for expression (31, 35), it is tempting to speculate that this specific sequence might play a more important role in ecdysterone-induced expression than one would a priori expect.

The identification of putative consensus sequences is surely an oversimplification of coordinate gene regulation, as protein-DNA interaction is far more complex than the simple recognition of one-dimensional DNA sequences. The comprehensive coordinate positive and negative regulation that must take place during the metamorphosis of an insect such as *D. melanogaster*, however, would seem to support the concept of an easily maintainable consensus sequence such as the one we have proposed. Since there is no direct evidence supporting the importance of this sequence, its role in ecdysterone-induced expression is extremely tentative. Its consistency with all results to date, however, is very significant.

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