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Sequence Requirement for Expression of the *Drosophila melanogaster* Heat Shock Protein *hsp22* Gene during Heat Shock and Normal Development

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A 14-base-pair sequence element present in almost all *Drosophila melanogaster* heat shock genes has been implicated in the heat inducibility of transcription. The *D. melanogaster* gene encoding the smallest heat shock protein, *hsp22*, contains within its 5′ flanking sequences three such repeats, two close to the transcription start site and a distally located third one 101 base pairs further upstream. Deletion analyses reveal that the 5′ flanking sequences required for full expression of the *hsp22* gene extend beyond the distal repeat. Deletion of the furthest upstream repeat results in a five to sixfold reduction of gene expression. The small heat shock genes are transiently expressed in the late third instar larval and early pupal stages without external stimulation. A deletion of 5′ flanking sequences to position −194, which includes two nucleotides of the distal heat shock element, has no effect on the developmental expression, whereas removal of an additional 18 nucleotides, including 12 nucleotides of the distal heat shock element, severely reduces developmental expression.

The heat shock genes of *Drosophila melanogaster* are coordinately induced as a response to the exposure to certain chemicals or high temperatures as well as during recovery from anoxia (for a recent review, see reference 33). All but one of these genes contain within their 5′ flanking regions one to four copies of the 14-base-pair (bp) sequence element, the heat shock element (HSE) CTnGAAnTTCnAG (5, 26, 34, 35). All of the four HSEs which are located upstream of the *hsp70* gene transcription start site specifically associate with the heat shock transcription factor (HSTF) in vitro (31, 46). The two contiguous HSEs closest to the TATA box bind HSTF cooperatively (46) and are indispensable and sufficient for full induction of in vitro-manipulated *hsp70* genes after reintroduction into the *D. melanogaster* genome, whereas the two HSEs located further upstream can be deleted without detectably affecting the heat inducibility of the genes (7).

The gene encoding the smallest heat shock protein, *hsp22*, has three HSEs upstream of the transcribed region, all of which fit the consensus sequence in 8 out of 10 positions (see Fig. 1). Two of them are closely spaced and only 26 and 46 bp from the TATA box, thus at relative positions similar to those of the two indispensable HSEs of the *hsp70* genes (see Fig. 7). A third element is found 147 bp upstream of the TATA box, again positioned like one of the two dispensable HSEs in the *hsp70* genes. To compare the sequence requirement for the full expression of the *hsp22* and *hsp70* genes during heat shock we truncated the 5′ nontranscribed region of the *hsp22* gene to various extents and reintroduced the manipulated genes into a fly strain which synthesizes an electrophoretic variant of hsp22 (hsp22^{var}). Direct comparison of the amount of protein encoded by the reintroduced and the endogenous genes reveals that full gene expression requires sequences that include all three HSEs.

The small heat shock genes are induced in the late third instar larval and early pupal stages (16, 25, 41, 42, 47). Sequences involved in the efficient developmental expression of the *hsp22* gene extend past the third HSE and thus overlap with the sequences required for heat induced expression.

MATERIALS AND METHODS

Protein labeling and analysis. Pairs of ovaries from 1 to 3-day-old flies were dissected and incubated in 10 μl of Shield medium lacking methionine. After 30 min at 35°C they were shifted to 37°C, 1 μl of [^{35}S]methionine (10 μCi) was added 10 min later, and the incubation was continued for 90 min. The medium was removed with a fine glass capillary, and the ovaries were dissolved either by boiling for 3 min in 15 μl of 40 mM Tris hydrochloride (pH 6.7)–2% sodium dodecyl sulfate–10% glycerol–5% 2-mercaptoethanol or in two-dimensional gel lysis buffer (30). Extracts of 1 to 10 μl were loaded onto 0.3-mm-thick, 21-cm-long 12.5% sodium dodecyl sulfate–polyacrylamide gels (21). The gels were run at 20 mA and dried, and autoradiograms were prepared by exposure to X-ray films (RX; Fuji). The autoradiograms were scanned with a Camag electrophoresis scanner and quantitated by measuring the peak heights.

Plasmid construction. Two different kinds of vectors were used for P factor-mediated germline transformation: pWP-6 (9) and car20 (38), which contain the white and the rosy indicator genes, respectively, inserted into a defective P element. Plasmid pWP-2 containing the white gene on a 12-kilobase (kb) *Eco*RI fragment cloned into a defective P element (9) was converted into pWP-6 by removing the *Eco*RI site 5′ to the white gene with the Klenow fragment of *Escherichia coli* DNA polymerase I. The *hsp22* gene, originally derived from the phage 179 (48) was contained in a 4.5-kb *Bam*HI fragment, which for other purposes had been subcloned in the *Bam*HI sites of a plasmid containing two inverted pUC9 polylinkers. The subcloned fragment was cut out between the *Eco*RI sites of the polylinkers and cloned into the *Eco*RI site of pWP-6.

The 3.9-kb *Acc*I-*Eco*RI fragment containing the *hsp22* gene (see Fig. 4) was subcloned into the *Acc*I site at position 2245 and the *Eco*RI site of pBR322. The resulting plasmid

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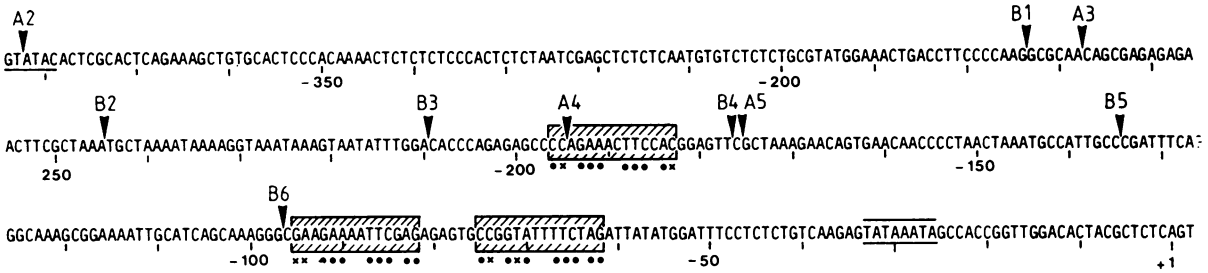

FIG. 1. Sequence upstream of the *hsp22* transcription unit (15, 43), with the deletion endpoints indicated by arrowheads. Boxed in are the HSE; the dots and crosses underneath point to the nucleotides which correspond and do not correspond to the consensus sequence, respectively. The *Acc*I site is underlined, and the TATA box is shown between lines. The transcription start site is indicated by +1.

was opened at the single *Acc*I site, treated with the exonuclease *Bal* 31 for different amounts of time, and cut with *Pst*I and *Bam*HI, and the ends were made blunt by treatment with T4 DNA polymerase. The mixture of the three fragments was ligated into the *Hpa*I site of car20 (38) treated with calf intestinal alkaline phosphatase. The same procedure, but omitting the *Bal* 31 digestion, resulted in the construct containing the intact *Acc*I-*Bam*HI fragment. Restriction at the *Pst*I site and removal of the 3′ overhangs were performed to inactivate the β-lactamase gene of pBR322. Constructs having the *hsp22* gene inserted in an orientation opposite to that of the indicator genes are designated with the letter A; the others are indicated with the letter B. *hsp22* is inserted downstream of the rosy gene and upstream of the white gene. The deletion endpoints of the A and B constructs are adjacent to the *Eco*RI and the *Hin*dIII sites in the polylinker of car20, respectively. The *Xba*I-*Eco*RI fragments of the A constructs and the *Xba*-*Hin*dIII fragments of the B constructs spanning the deletion endpoints were cloned into the *Xba*I-*Eco*RI sites of M13mp11 and the *Xba*I-*Hin*dIII sites of M13mp10, respectively, and were sequenced by the method of Sanger et al. (40).

Fly strains and P factor-mediated transformation. The genotype of the fly strain G, which was used as the recipient for transformation with rosy *hsp22* P elements, is *hsp22*^{var} *th st cu ry*^{506}/*hsp22*^{var} *th st cu ry*^{506}. The recipient strain for transformation with the white *hsp22* P element is homozygous for the *hsp22*^{var} allele and the mutation w^1. Embryos were injected as described by Rubin and Spradling (37, 44) with 300 μg of the plasmid containing the sequence to be integrated into the fly genome per ml and 50 μg of the helper plasmid pπ25.1 (44) (white *hsp22* P element) or pπ25.7 *w/c* (18) (rosy *hsp22* P elements) per ml. The DNA was purified by preparing a cleared supernatant as described by Clewell and Helsinki (6) followed by banding in CsCl-ethidium bromide gradients. Flies arising from injected embryos were backcrossed to the recipient fly strains once and subsequently crossed to the balancer stocks described by Hultmark et al. (14) to obtain homozygous strains. The strain A1.1 obtained from transformation with the white *hsp22* P element was made homozygous by continuous inbreeding.

Southern hybridization. DNA from flies was prepared essentially as described by Bender et al. (4). Extraction buffer (300 μl; 0.1 M Tris hydrochloride [pH 9.0], 0.1 M NaCl, 0.2 M sucrose, 50 mM EDTA, 0.5% sodium dodecyl sulfate) and 12 μl of diethylpyrocarbonate were added to 20 flies frozen in liquid nitrogen in an Eppendorf tube and homogenized with a glass pestle. An additional 300 μl of extraction buffer was added, and the homogenate was first incubated at 65°C for 30 min and then on ice for 30 min after the addition of 120 μl of 8 M potassium acetate. Samples of 500 μl of supernatant were collected after 5 min of centrifugation in an Eppendorf centrifuge, and nucleic acids were precipitated in 50% ethanol for 5 min at room temperature. Two fly equivalents of the DNA were cut with *Sal*I and treated with 10 μg of RNase A per ml for 1 h, and the fragments were separated on a 0.7% agarose gel. Transfer of the DNA to nitrocellulose filters and hybridization were done as described by Maniatis et al. (24). The probe used for hybridization was the plasmid pD19.1, containing the *hsp22* gene including 3.2 kb of the 5′ flanking sequence (14).

Analysis of pupal extracts. To accurately stage pupae, we marked the position of white prepupae on the glass vials not later than 30 min after the onset of pupation. Five to 10 pupae were homogenized in 10μl of two-dimensional gel sample buffer (30) per pupa in an Eppendorf tube with a glass pestle. The extracts were centrifuged for 5 min, and the supernatants were stored at −20°C. Samples (10 μl) of extracts were applied to two-dimensional gels (30), and the gels were silver stained as described by Morrissey (28).

Primer extension. Pupae were staged as described above and frozen in a dry ice-ethanol mixture. Flies were heat shocked for 60 min at 37°C and frozen in liquid nitrogen. RNA was prepared as described previously (20). For the preparation of single-stranded, ^{32}P-labeled primer DNA we extended a synthetic 15-mer oligonucleotide (BioLabs) with the DNA polymerase I Klenow fragment in the presence of [^{32}P]dATP on M13mp11 phage DNA containing a 280-bp *Hin*dIII-*Xba*I fragment from the promoter and leader region on the *hsp22* gene (14). The extension product was cut with *Xmn*I, and the 101-nucleotide fragment was purified on a sequencing gel (39). Hybridization to RNA and the primer extension reaction were done as described by Hultmark et al. (14).

RESULTS

Construction of *hsp22* gene-containing P elements and germline transformation. We constructed a series of plasmids, suitable for P factor-mediated transformation, containing the gene encoding the smallest heat shock protein, *hsp22*, and various lengths of 5′ flanking sequence. The initial construct, A1 (see Fig. 4), contains 3.2 kb of sequences upstream of the transcriptional start site and was inserted as a 4.3-kb *Bam*HI fragment next to the white gene, which is flanked by the two halves of a defective P element in the plasmid pWP-6 (9). The white sequences in this plasmid were previously shown to allow rescue of the white mutant phenotype when inserted into the genome by P factor-mediated transformation (9). White-eyed flies homozygous for the mutation white^1 (w^1) and the *hsp22* allele encoding a slowly migrating variant protein (*hsp22*^{var}) were transformed with pWP-6 containing the *hsp22* gene. The occurrence of

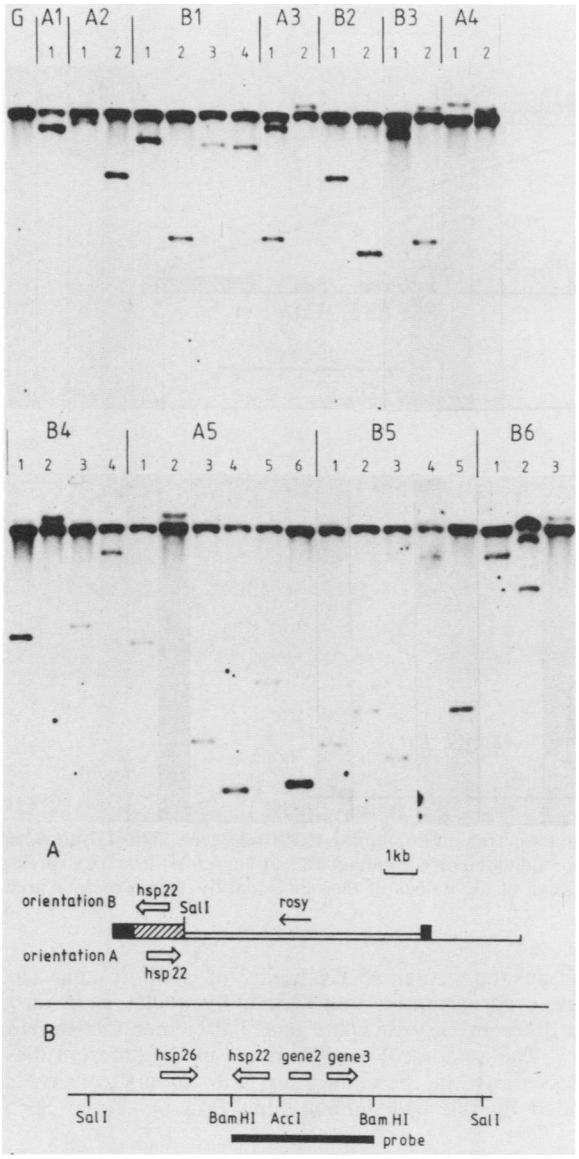

FIG. 2. Genomic Southern blots of *Sal*I-restricted DNA from transformed fly strains and recipient strain G. The plasmid used for transformation is shown in the lower part (panel A), with filled, striped, and open bars representing the P element, the *hsp22* gene, and the rosy gene, respectively. The arrows indicate the direction of transcription of the rosy and the *hsp22* genes. The *Sal*I site flanking the *hsp22* gene is unique in the plasmid. Panel B shows the genomic organization of the region containing the *hsp22* gene. The 4.3-kb DNA fragment used as the probe for the Southern hybridization experiment is indicated by the filled bar. Genes 2 and 3 are related to the four major small heat shock genes and are weakly heat inducible (3).

red-eyed transformed flies was very low (5%), probably due to the large size of the DNA to be integrated (17 kb); therefore we used the shorter xanthine dehydrogenase (rosy) gene in the plasmid car20 (38) as an indicator for successful transformation in subsequent experiments.

We then reduced the extent of the 5′ flanking sequences by first trimming the DNA to the *Acc*I site 382 bp upstream of the transcriptional start site. Further removal of flanking sequences down to 96 bp from the transcription initiation site was achieved by digestion with the exonuclease *Bal* 31. The

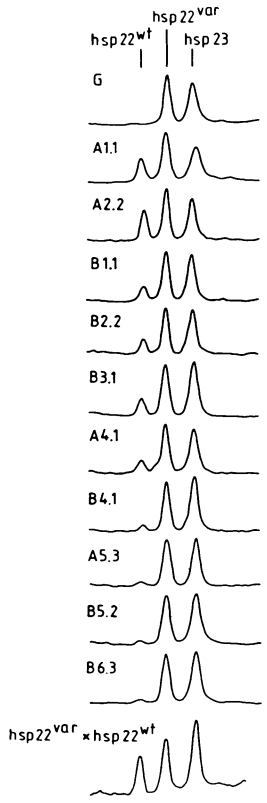

FIG. 3. Expression of *hsp22* in different transformed fly strains. Shown are densitometric tracings of the lower part of an autoradiogram. The proteins of only one representative fly stock for each deletion as well as of the recipient fly strain and a strain heterozygous for the wild type and the *hsp22*^{var} genes are shown.

truncated DNA fragments were inserted into the unique *Hpa*I site of the plasmid car20. The deletion endpoints were determined by sequencing (Fig. 1). Constructs with the rosy and the *hsp22* genes in the same orientation are designated B1 to B6, and those in the opposite orientation are designated A2 to A5. The rosy *hsp22* elements were used to transform flies homozygous for the rosy mutation *ry*^{506} and the *hsp22* variant allele *hsp22*^{var}. Transformed fly strains were made homozygous for the inserted genes by crossing them to the appropriate balancer stocks.

Determining the copy number of the inserted genes. We performed a genomic Southern hybridization experiment to determine the number of integrated copies. The restriction enzyme *Sal*I cuts in the polylinker of car20 right next to the inserted *hsp22* sequence in the rosy *hsp22* P elements but has no further recognition site in the *hsp22* sequences (Fig. 2). For each inserted copy one expects one *Sal*I fragment with a minimal length of 1.5 to 4.7 kb, depending on the construct. The endogenous *hsp22*^{var} gene yields a 12-kb *Sal*I fragment. In 32 cases one additional band was detected, and in 3 cases (A3.1, B3.2, and B6.2) two additional bands were detected. Consequently, to compare the amounts of accumulated hsp22^{wt} in strains with different rosy or white *hsp22* P elements we divided the values obtained from the strains with two integrated copies by two (see below). The part of the probe homologous to the integrated sequences containing the *hsp22* gene is different for each construct and the resident *hsp22* variant gene, which explains the variable intensity of the hybridization signals.

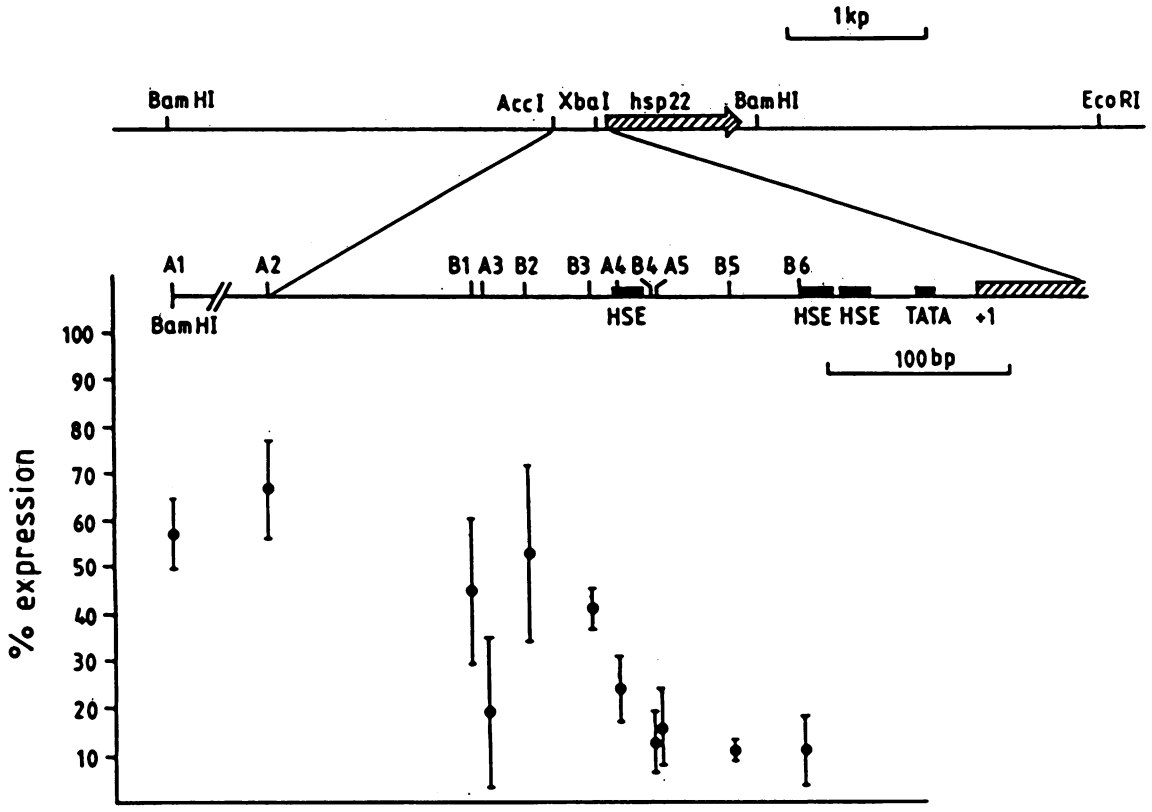

FIG. 4. Quantitation of *hsp22*^{wt} expression. The top line represents the genomic region containing the *hsp22* gene. The 4.3-kb *Bam*HI fragment was used for the A1, K, and T/2 constructs. The deletion endpoints are indicated on the expanded map, and their level of expression as compared with that of *hsp22*^{var} is shown underneath. The values are the mean of the values of the individual fly strains as indicated in Table 1.

Expression of the transformed *hsp22* genes during heat shock. Ovaries of transformed flies were dissected and exposed to heat shock temperatures, and the proteins were labeled with [^{35}S]methionine. After separation of proteins on sodium dodecyl sulfate-polyacrylamide gels, autoradiograms were prepared and densitometrically traced to allow quantitation of the proteins. Examples of such tracings from different representative transformed fly stocks, each harboring a different rosy or white *hsp22* P element, is shown in Fig. 3. The amount of hsp22^{wt} made in the different flies is expressed as the percentage of the amount of hsp22^{var} encoded by the endogenous gene (Fig. 4; Table 1). We

TABLE 1. Expression of *hsp22*^{wt}

Strain	% hsp22^{var}[a]	Distance[b]	Strain	% hsp22^{var}	Distance	Strain	% hsp22^{var}	Distance
A1.1	52		A3.1	60[c]	262 bp	A5.1	9	
			A3.2	7		A5.2	16	
K/1	64					A5.3	15	175 bp
K/2	66	3.2 kb	B2.1	65	244 bp	A5.4	30	
K/6	53		B2.2	38		A5.5	22	
						A5.6	7	
T/2	45		B3.1	40	209 bp			
			B3.2	89[c]		B5.1	10	
A2.1	75	382 bp	B3.3	36		B5.2	9	
A2.2	60					B5.3	13	134 bp
			A4.1	29	194 bp	B5.4	13	
B1.1	39		A4.2	19		B5.5	9	
B1.2	55	273 bp						
B1.3	60		B4.1	18	176 bp	B6.1	20	
B1.4	25		B4.2	20		B6.2	21[c]	
			B4.3	6		B6.3	11	96 bp
			B4.4	9		B6.4	2	

[a] The values represent the average of two experiments and are expressed as the percentage of the amount of hsp22^{var}.
[b] Indicated in base pairs or kilobases are the distances of the deletion endpoints from the transcription start site.
[c] Values from strains which are tetraploid for the rosy *hsp22* P element. For calculation of the mean values as plotted in Fig. 4, they were divided by 2.

included the values obtained with the constructs K and T, described by Hultmark et al. (14), both of which contain 3.2 kb of flanking region as A1.1 (Table 1). In addition they have a 111-bp DNA fragment inserted in the 3′ noncoding trailer region, and one of them has an additional *Kpn*I linker inserted in the leader region (K). By calculating the efficiency of *hsp22*^{wt} expression one has to take into account that the *hsp22*^{wt} gene is not expressed as well as the variant allele even in its natural location. The hsp22^{wt} level in heterozygous strains amounts to only 75% of the hsp22^{var} level (Fig. 3).

hsp22 genes with 3.2 kb to 209 bp of natural flanking sequences are efficiently expressed (Fig. 4, A1 to B3; Table 1). The only notable exception is strain A3.2. Its extremely low level of expression might be due to inhibitory effects of host flanking sequences (see below). Further deletions to position −176 (Fig. 4, B4) result in a fivefold drop of gene activity. Additional truncations to position −96 (Fig. 4, B6) do not reduce the expression any further. Ayme et al. (2) have shown that the deletion of the proximal HSEs abolishes expression of the *hsp22* gene in COS cells altogether. We do not know whether the same holds true for *D. melanogaster*, because the sequence requirements for heat shock gene expression in COS cells and in *D. melanogaster* are not always identical (see Discussion). This experiment documents the presence of a sequence element between the deletion endpoints B3 and B4 within a 33-bp stretch of DNA which is important for full gene activity. The 14 bp of the distal HSE localized within this region is likely to be the crucial sequence.

Having the transformed gene in a strain which synthesizes an electrophoretic variant of hsp22 allows accurate quantitation of its product, because experimental variations should affect both genes in the same way and thus become irrelevant. However, expression of the same gene in different transformed strains varies considerably, precluding very precise measurements of gene activity. We attribute this variation to the influence of the different *D. melanogaster* sequences flanking the integrated genes. This position effect is observed independently of the orientation of the *hsp22* gene. Even 3.2 kb of 5′ flanking sequences is insufficient to buffer the influence of neighboring host sequences on the expression of the transformed genes. A similar position effect has been observed by others (11, 45). Detection of subtle alteration in promoter strength therefore requires the analysis of a large number of transformed lines.

Deletion of the 5′ flanking region does not result in altered transcription initiation sites. The amount of hsp22^{wt} protein synthesized in the different transformed fly strains can be taken as a measure of promoter strength only if the same kind of mRNA is being copied from the genes with 5′ deletion, since different transcripts might differ in their stability or translatability. We do not have an easy means to selectively detect mRNA copied from the transformed *hsp22* gene; therefore putative *hsp22* mRNAs with different 5′ ends have to be detected against the background of mRNA from the endogenous *hsp22*^{var} gene. Primer extension experiments were performed with RNA isolated from five transformed strains and the recipient strain G. A 101-nucleotide primer fragment complementary to the 3′ half of the leader region should yield a 255-nucleotide DNA fragment if extended to the 5′ end of wild-type *hsp22* mRNA. The main extension product has the expected size of 255 nucleotides (Fig. 5). We observe no longer products but a large number of faint, smaller ones. If the genes with deletions in their flanking region gave rise to mRNAs with different 5′ ends,

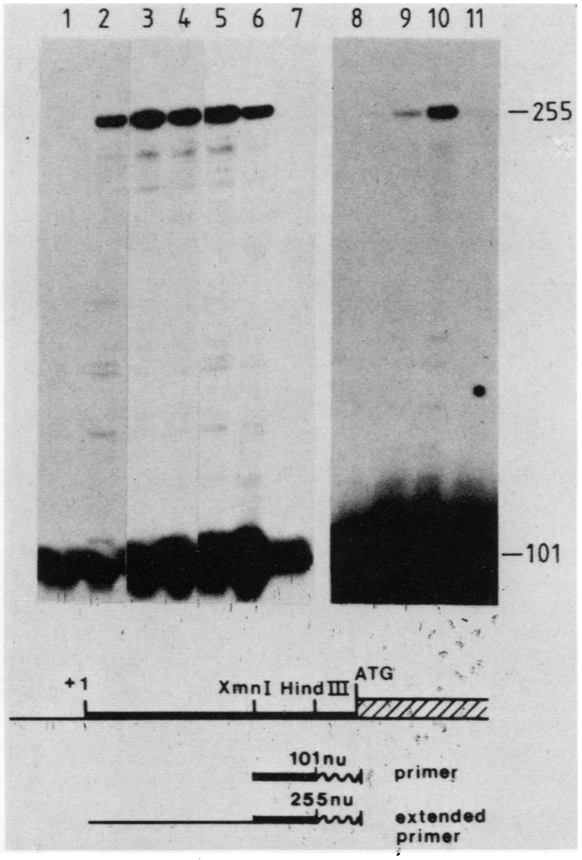

FIG. 5. Primer extension experiment. A 101-nucleotide single-stranded DNA primer (lane 7) (20,000 cpm, Cerenkov) was hybridized to 6 μg (lanes 1 through 6) or 20 μg (lanes 8 through 11) of RNA and extended with reverse transcriptase. Sequencing reactions of a nonrelated DNA fragment run in parallel were used as molecular weight markers (data not shown). RNA was isolated from A2.1 flies not subjected to a heat shock (lane 1), from flies incubated for 45 min at 37°C before harvesting of strains A2.1 (lane 2), B3.2 (lane 3), A5.4 (lane 4), B6.2 (lane 5), and the recipient strain G (lane 6), or from A2.1 white prepupae (lane 8) and 1-day-old (lane 9), 2-day-old (lane 10), and 3-day-old (lane 11) pupae. The lower panel shows part of the *hsp22* gene with the coding and noncoding transcribed sequences indicated by striped and filled bars, respectively. The wavy line at the 3′ end of the primer represents sequences derived from phage M13.

bands absent from the extension products of mRNA isolated from the recipient fly strain were to be expected. Their intensity should reflect the level of hsp22^{wt}, which for the different strains is between 21 and 89% of the hsp22^{var} level (Table 1) (notice that the strains B6.2 and B3.2 have two rosy *hsp22*^{var} P elements inserted). No such bands are present, and we conclude therefore that the upstream deletions did not result in altered transcription initiation sites. This result suggests that the same kind of mRNA is made from the deletion genes and the resident gene, which would imply that the amount of hsp22 is a measure for promoter strength. We did not, however, rigorously exclude the possibility of posttranscriptional effects as the 5′ deletion might affect the 3′ ends of the mRNA.

Expression during normal development. The small heat shock genes are transiently activated during normal development, whereas the other heat shock genes are kept inactive. To determine the sequence requirement for develop-

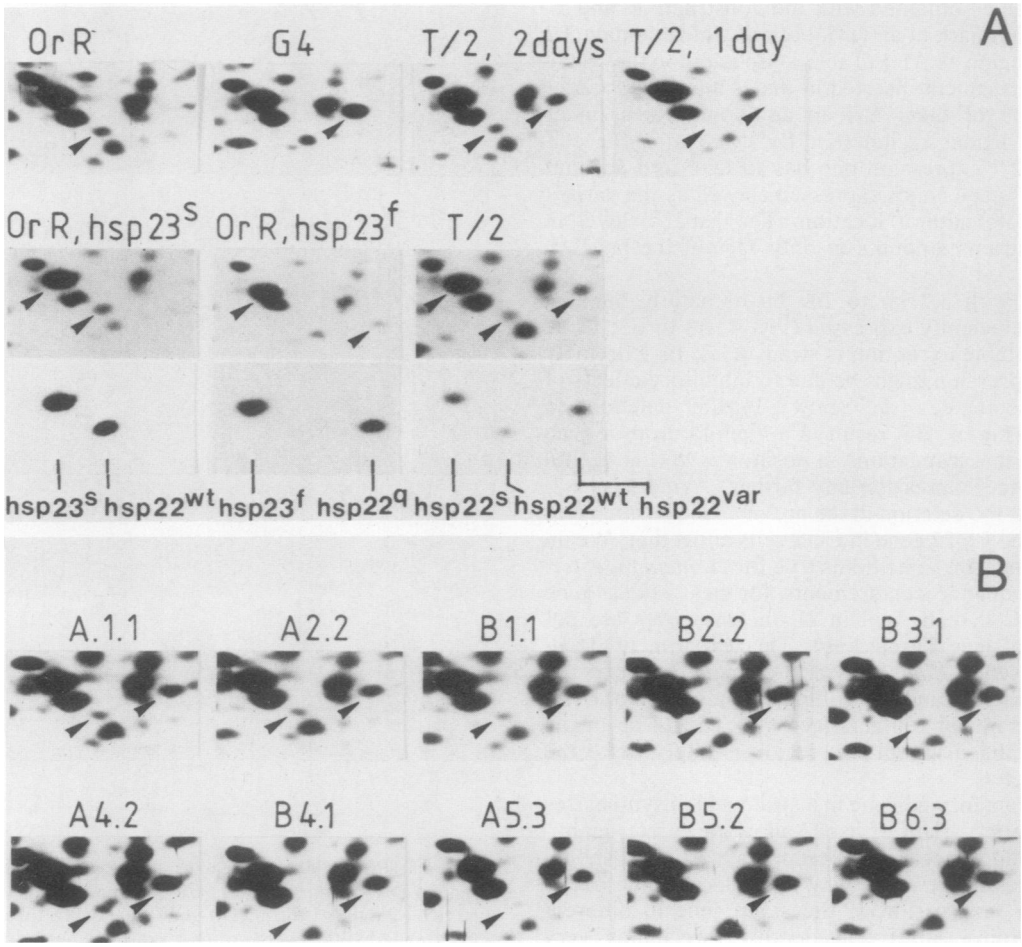

FIG. 6. Expression during normal development. The relevant regions of silver-stained, two-dimensional gels or autoradiograms thereof (panel A, third row) are shown. Three-day-old pupae (unless otherwise indicated) were homogenized, and protein extracts from one-half (panel A, second row) or one pupa were analyzed. The extract of one pair of ovaries, labeled for 2 h at 37°C, was mixed with pupal extracts (panel A, second row), and an autoradiogram was prepared from the dried gels (panel A, third row). The Oregon-R (OrR) strains used in this experiment carry cloned third chromosomes with the wild-type genes *hsp23*^{S} and *hsp22*^{wt} or the variant genes *hsp23*^{f} and *hsp22*^{q}. The arrowheads point to *hsp22*^{wt} and *hsp22*^{var} (panel A, first row; panel B) and the different electrophoretic forms of hsp22 and hsp23 (panel A, second row).

mental expression, we analyzed what effect the various deletions of the *hsp22* gene 5′ flanking region exerted on the expression in early pupae. Among the four major small heat shock proteins, hsp22 is the one which accumulates the least during development (3, 25, 47). Nevertheless, it can easily be detected on silver-stained two-dimensional gels. Protein spots on such gels could be assigned to hsp22^{wt} and hsp22^{var} with the help of the wild-type strain Oregon R and the transformation recipient strain G, which synthesize only hsp22^{wt} and hsp22^{var}, respectively (Fig. 6A). Primer extension experiments performed with RNA isolated from non-heat-shocked, accurately staged prepupae and pupae revealed that the level of *hsp22* mRNA reaches the peak 2 days after pupariation and rapidly falls thereafter (Fig. 5, lanes 8 through 11). Consistent with this result, hsp22 only starts to accumulate between 1 and 2 days after the onset of pupation (Fig. 6A, compare T/2 on days 1 and 2). hsp23, on the other hand, is much more abundant and can be detected already in third-instar larvae (data not shown). Its unambiguous localization on two-dimensional gels again was possible with the help of an electrophoretic variant. One of our Oregon R strains is polymorphic for the *hsp23* gene. To determine the position of hsp23 we cloned the chromosome carrying the *hsp23* variant gene (*hsp23*^{f}). It turned out that this chromosome also carries an *hsp22* variant gene (*hsp22*^{Q}) whose product almost comigrates with hsp22^{wt} on sodium dodecyl sulfate-polyacrylamide gels but not on two-dimensional gels and therefore represents a different variant than the one present in strain G. Comigration of proteins labeled with [^{35}S]methionine during heat shock confirmed the assignments of the various heat shock proteins (Fig. 6A).

The protein patterns of pupal extracts from the different transformed strains are shown in Fig. 6B. Deletions down to −194 (A1, A2, B1, B2, B3, and A4) did not substantially affect *hsp22* expression. Further deletion to −176 (B4) resulted in a strong reduction of the expression, whereas the two longest deletions, B5 and B6, reduced expression to undetectable levels. No hsp22 spot was seen even on gels where twice the amount of extract was applied from strain B6.2, which carries two rosy *hsp22* P element copies per haploid genome (data not shown). The 60 bp between −134 and −194 and in particular the 18 bp between −194 and −176 are therefore crucial for the expression of the *hsp22* gene during development.

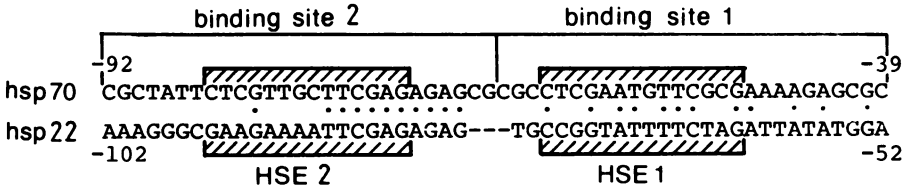

FIG. 7. Comparison of the DNA regions containing the proximal HSEs of the *hsp70* and *hsp22* genes. The HSEs are emphasized with hatched boxes, and the *hsp70* gene sequence protected in DNase I footprint experiments described by Topol et al. (46) are indicated as binding sites 1 and 2. Sequence homologies are marked with dots. The numbers indicate the distances from the transcription initiation site.

DISCUSSION

Sequence requirement for heat induction. Our experiments demonstrate that sequences between −209 and −176 enhance the expression of the *hsp22* gene during heat shock approximately fivefold. Located within these 33 bp is the distal HSE. It is likely to determine increased promotor strength by interacting with the HSTF. However, we cannot exclude the possibility that another transcription factor can bind to other sequences within that region.

Sequences including only two of the four HSEs were shown to be sufficient for full heat activation of the *hsp70* genes in flies (1, 7). Cooperative binding of the HSTF to these two contiguous HSEs in vitro has recently been demonstrated (46). The *hsp22* gene also contains within its 5′ flanking region two contiguous HSEs at a similar distance from the TATA box. As shown here they are, however, not sufficient for full activation of the gene. Interactions between the HSTFs, binding to the two HSEs, might account for the full activation of the *hsp70* promoter. The same putative interactions are unlikely to occur upstream of the *hsp22* gene, since the distance between the two HSEs is 3 bp shorter, which would shift the relative position of bound HSTFs by about one-third of a helical turn (Fig. 7). The closer proximity of the HSEs might even preclude binding of a second HSTF altogether. Alternatively, interactions between HSTF and factor B bound to or in the vicinity of the TATA box (31, 32) might occur and allow for full promoter activation. Again, the additional 11 bp separating the two binding sites upstream of the *hsp22* gene might not allow for the same protein interactions.

It has recently been reported that a deletion to −100 of the *hsp22* gene, leaving only the two proximal HSEs intact, does not result in a noticeable reduction of the promoter strength (2). This conflicting conclusion was derived from experiments performed in COS cells. COS cells, however, do not seem to mimic accurately the situation in flies in all cases. Expression from the *hsp70* promoter requires only one of the two proximal HSEs (26, 35); the expression from the *hsp27* promoter was low (36) or undetectable (2) and expression from the *hsp23* promoter was undetectable (36) or constitutive (2), in contrast to results obtained from transfection experiments with the *D. melanogaster* cell line S3, in which efficient expression from both promoters in response to heat shock was demonstrated (27).

Sequence requirement for developmental expression. All of the small heat shock genes are activated around the onset of pupation (16, 25, 41, 42, 47). At that time the titer of the molting hormone β-ecdysone reaches a peak (13), and it has been proposed that the heat shock gene induction is hormonally regulated (16, 25, 41, 42, 47). Treatment of *D. melanogaster* tissue culture cells with β-ecdysone indeed induces expression of the small heat shock genes (16, 17, 22, 47). We have detected low levels of *hsp22* mRNA in white prepupae and a substantial transient accumulation 2 days later (Fig. 6), coinciding with the main peak of hormone release (13). The *hsp22* gene might be directly induced through the binding of the β-ecdysone–receptor complex. Such a protein-DNA interaction would most likely occur within the 60 bp between −134 and −194, since the deletion of this region abolishes *hsp22* expression in pupae. Alternatively, the activation of the small heat shock genes might be indirect. It has been suggested that the heat shock response is triggered by the accumulation of abnormal proteins (8, 10). Munro and Pelham have recently proposed a model that could explain this phenomenon (29). They suggested that the HSTF is a very unstable protein which under normal conditions is subject to rapid ubiquitin-mediated degradation. An increase of protein degradation due to denaturation of normal proteins at elevated temperatures might limit the free pool of ubiquitin, thus ensuring a longer half-life of the HSTF, which in turn can bind to and activate the heat shock genes. Our experiments demonstrate that efficient expression of the *hsp22* gene during development depends on sequences between the deletion endpoints A4 and B4. This 18-bp stretch of DNA contains almost all of the distal HSE. (The 5′ most sequence CC of the distal HSE [Fig. 2] has been replaced in the deletion A4 by TT, which does not change the overall fit to the consensus HSE sequence TC. That might explain why the A4 deletion does not result in reduced expression during development.) It is therefore plausible that the HSTF is also involved in gene induction during development. Following the model of Munro and Pelham, it is conceivable that the massive protein degradation during histolysis in early pupae might allow for a high enough HSTF concentration. One would have to postulate a lower threshold level of HSTF for the induction of the small heat shock genes, which is consistent with the observation that they are induced at lower temperatures (23), to account for their selective activation during development. Additional factors might, however, also be involved. Deletion of the distal HSE to −176 (B4) reduces developmental expression drastically but not completely. Further deletion to −134 (B5), which does not result in additional reduction of the expression during heat shock, abolishes developmental expression altogether. Thus the sequence requirement for the two modes of gene activation might not be completely congruent. A further indication that the mechanism of heat shock gene activation during heat shock and development are not identical comes from the fact that hsp23 accumulates to much higher levels than hsp22 during development only (compare Fig. 2 and 6). On the other hand, hsp22 is most abundant among the heat shock proteins in the indirect flight muscle of some flightless mutants (12, 19). These mutants express the heat shock genes constitutively as a consequence of a lesion in the gene which encodes the actin that is specifically expressed in the indirect flight muscles (12, 19). Apparently there is not one unique mechanism of induction common to

all small heat shock genes. Expression of the *hsp26* and *hsp27* genes but not of the *hsp22* and *hsp23* genes during oogenesis represents an extreme example of noncoordinate expression of the small heat shock genes (49). The three genes which are interspersed among and related to the small heat shock genes provide yet another example (3, 42). Two of them (1 and 3, following the nomenclature of Ayme and Tissières [3]) are expressed efficiently during development but only weakly during heat shock, whereas gene 2 is weakly expressed during heat shock and in embryos but not in pupae. In addition, gene 1 is also active in young adult flies. Furthermore the extent of heat inducibility of gene 3 is dependent on the developmental stage. Hence, there must be multiple mechanisms of induction of the small heat shock genes which clearly deserve further investigation.

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