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Genetics

Four heat shock proteins of *Drosophila melanogaster* coded within a 12-kilobase region in chromosome subdivision 67B

(cloned DNA/heat shock)

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ABSTRACT **Unique coding sequences for four heat shock proteins of *Drosophila melanogaster*, hsp 28, hsp 26, hsp 23, and hsp 22, are clustered in a 12-kilobase interval at chromosome subdivision 67B. The four genes are not transcribed in the same direction and each gives rise to a separate messenger RNA, with no indication of intervening sequences. Including the present results, the genes for all seven major heat shock proteins of *D. melanogaster* are now cloned and are found to exhibit a variety of patterns of organization at the five loci they occupy.**

Temperature elevation and certain other treatments induce in *Drosophila melanogaster* the vigorous synthesis of seven proteins of unknown function, referred to as heat shock proteins [reviewed by Ashburner and Bonner (1)]. Two of these, with molecular weights 83,000 (hsp 83) and 68,000 (hsp 68), are coded by unique sequences in polytene chromosome regions 63BC and 95D, respectively, where heat shock induces prominent puffs (2). A third heat shock protein, hsp 70, is coded at two other heat shock puff sites, 87A and 87C, with two copies of the hsp 70 coding sequence at the former and three at the latter (2, 3).

Coding sequences for three additional heat shock proteins, hsp 23, hsp 26, and hsp 28, have been reported to lie within a single *Bam*HI restriction endonuclease fragment derived from the heat shock puff site at 67B. This is consistent with linkage studies of alleles affecting the mobility of the proteins and with results from hybridization *in situ* by size-fractionated heat shock mRNA (4–6). The results we report here extend the analysis of this locus and show that unique coding sequences for all four of the smaller heat shock proteins, hsp 22, hsp 23, hsp 26, and hsp 28, are clustered within an interval of 12 kilobases (kb) in chromosome subdivision 67B. The four genes are not transcribed in the same direction, and each gene specifies a separate mRNA with no indication of intervening sequences.

MATERIALS AND METHODS

Materials and methods not referenced in the text are described below or have been described earlier (2, 7). The clone λ88 was selected from a λ Charon 4 library of *D. melanogaster* Canton S embryo DNA generously made available by T. Maniatis (8). Whole-cell heat shock RNA was prepared from 12- to 18-hr embryos grown at 25°C, heat shocked at 36°C for 60 min, and frozen in liquid nitrogen. Thawed embryos were dechorionated in 2.5% sodium hypochlorite/1% NaCl/1% Triton X-100 for 2 min, washed with water, and lysed in a Dounce homogenizer in 7.0 M urea/2% sodium dodecyl sulfate/0.35 M NaCl/10 mM Tris·HCl/1 mM EDTA, pH 8. The mixture was extracted three times with phenol/chloroform/isoamyl alcohol (50:50:1, vol/vol) and RNA was precipitated with ethanol and centrifuged. The pellet was resuspended in 10 mM Tris·HCl, pH 7.4/10 mM NaCl/10 mM $MgCl_2$/1 mM $CaCl_2$, digested with RNase-free DNase (9), extracted with phenol, precipitated with ethanol, dissolved in H_2O, mixed with 3 vol of 4 M sodium acetate, pH 6.0, kept on ice 2 hr, and centrifuged 30 min at 4°C at 16,000 × *g*. The precipitated RNA was dissolved in H_2O, precipitated with ethanol, and kept at −70°C.

Plasmid restriction fragments were labeled at the 3′ end by using nucleoside [α-^{32}P]triphosphates and 10 units/ml of *Escherichia coli* DNA polymerase I Klenow fragment (2). Flush-ended restriction fragments were incubated with exonuclease III (gift of W. McClure) for 20 min at 5°C (10) before 3′-labeling. Restriction fragments were 5′-labeled by using [α-^{32}P]ATP and polynucleotide kinase (11). ^{32}P-Labeled cDNA was copied from RNA according to Efstratiadis *et al.* (12). Work with recombinant phages and plasmids was done under P2 + EK1 conditions.

RESULTS

A library of *D. melanogaster* embryo DNA cloned in λ Charon 4 was screened by plaque hybridization with ^{32}P-labeled cDNA copied from the mRNA of heat-shocked *Drosophila* tissue culture cells. Positive clones were purified and rescreened by hybridization with ^{32}P-labeled cDNA made from 10–12S heat shock mRNA, the fraction in which messenger activity for the smaller heat shock proteins resides (6, 13). DNA from the clone that hybridized most strongly, designated λ88, was labeled with [^{3}H]thymidine by nick translation and hybridized *in situ* to salivary gland polytene chromosomes. As shown in Fig. 1, intense labeling was found in subdivision 67B. No other site was labeled above the background.

Fig. 2 presents a restriction map of the 16.8-kb *Drosophila* segment of λ88, deduced from the sizes of fragments produced

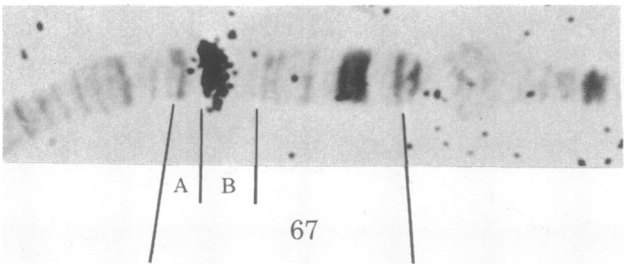

FIG. 1. Hybridization *in situ* by ^{3}H-labeled λ88 DNA at salivary gland polytene chromosome subdivision 67B of *D. melanogaster*. Specific activity 2 × 10^8 dpm/μg, 3-day exposure.

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Abbreviations: kb, kilobase(s); hsp, heat shock protein.

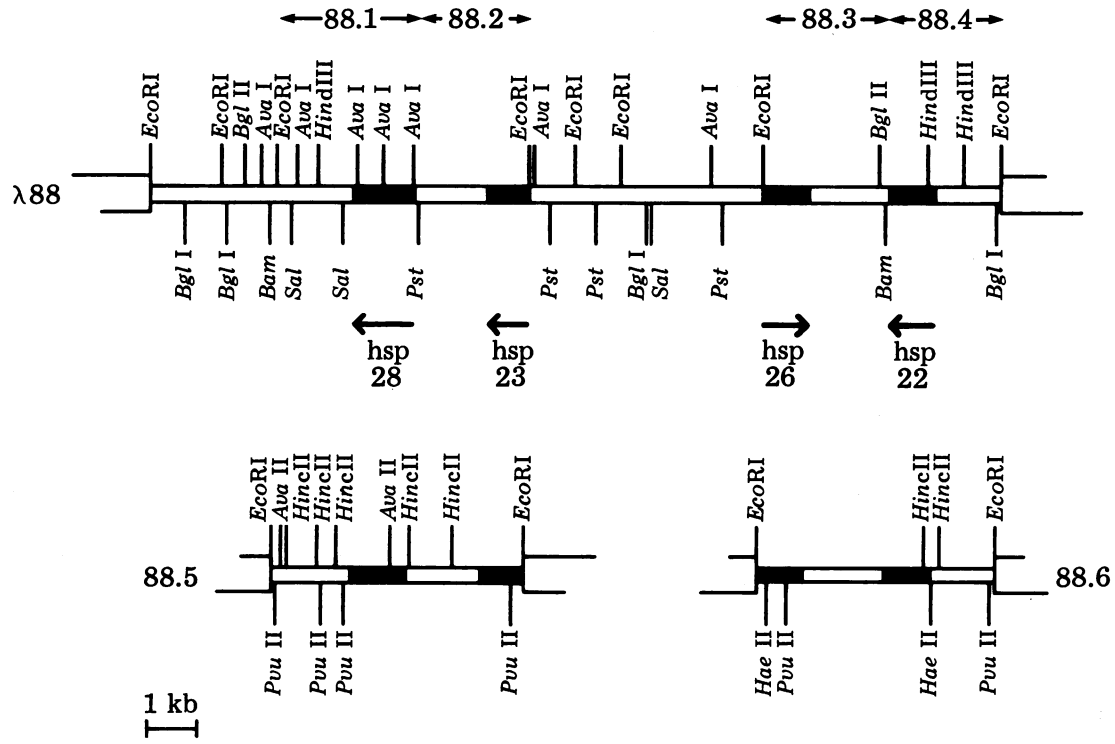

FIG. 2. Restriction map of λ88 and subclones 88.5 and 88.6. The four restriction fragments cloned in 88.1–88.4 are also indicated. *Ava* II sites are mapped only in the region of 88.5 common to 88.1. Filled sections depict homology to heat shock RNA and arrows show the direction of transcription.

by single and double digestion with the indicated restriction enzymes. Additional restriction sites are mapped on two fragments subcloned from λ88 into pBR322, designated 88.5 and 88.6. The locations of four smaller fragments subcloned in pBR322, 88.1–88.4, are also indicated.

Regions homologous to RNA were mapped on the basis of their resistance to S1 nuclease after hybridization with whole cell RNA from heat shocked embryos, using the procedure of Berk and Sharp (14). For example, 88.3 gives rise to a single S1-resistant fragment of length 0.95 kb. Digestion of the DNA with *Pvu* II before hybridization with RNA produces S1-resistant fragments of length 0.60 and 0.35 kb. Thus, 88.3 contains a single region homologous to heat shock RNA, with a *Pvu* II site located 0.60 kb from one end. There is a single *Hae* II site in the 88.3 *Drosophila* segment, 0.50 kb to the left of the *Pvu* II site. Hybridization of a *Hae* II digest with heat shock RNA

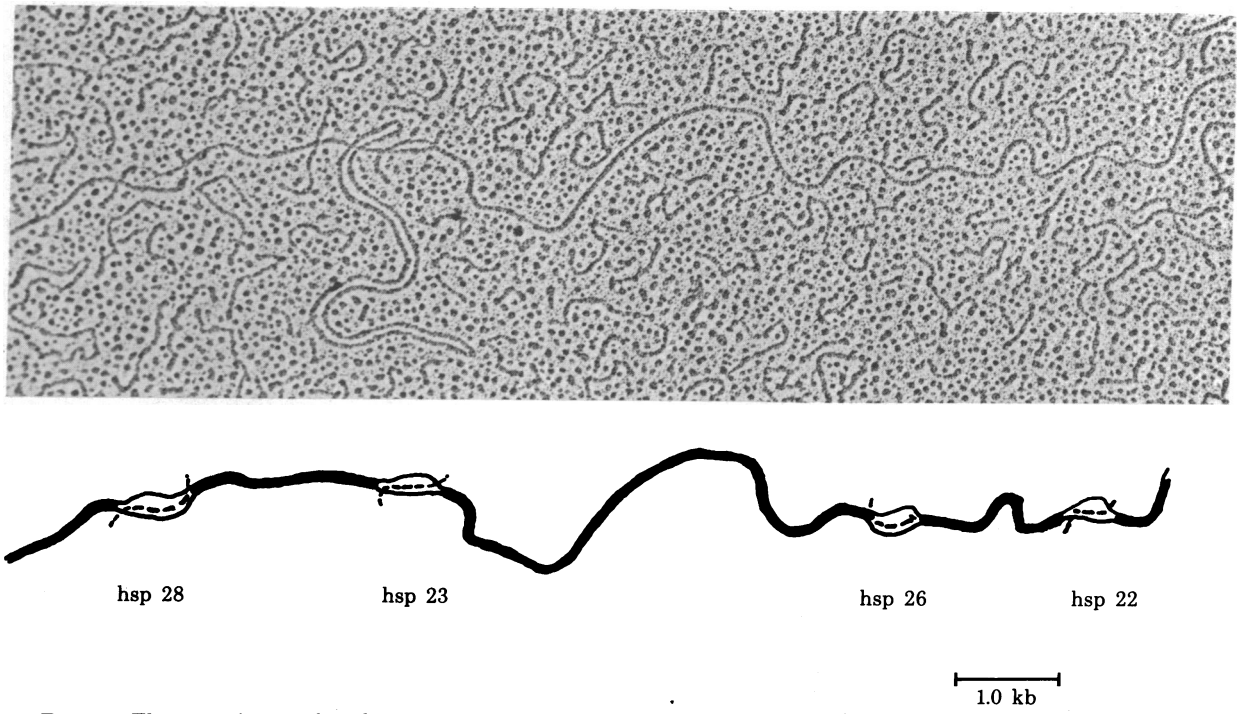

FIG. 3. Electron micrograph and schematic interpretation of RNA·DNA hybrid of λ88 and whole cell heat shock RNA.

gives rise to a 0.75-kb S1-resistant fragment, positioning the region of RNA homology about the *Pvu* II site as shown in Fig. 2. Each of the other three small subclones also yielded only a single S1-resistant fragment, positioned as shown in the figure. The lengths of the regions homologous to heat shock RNA on subclones 88.1–88.4 are 1.15, 0.85, 0.95, and 0.95 kb, respectively. Hybridization of various restriction digests of λ88 itself with whole cell heat shock RNA gave no indication of homology to RNA except in the regions shown in Fig. 2.

The regions of λ88 homologous to heat shock RNA were also mapped by electron microscopic examination of DNA·RNA hybrid molecules, under conditions favoring the formation of R-loops (15). Such a hybrid is shown in Fig. 3. Four R-looped regions are typically found, with average spacings in agreement with those found by restriction mapping and analysis of S1-resistant fragments.

The four regions of λ88 homologous to RNA were identified by hybrid-selected translation *in vitro* (16) as coding sequences for the four small heat shock proteins. As shown in lane 1 of Fig. 4, RNA from heat-shocked embryos is translated *in vitro* to give the four small heat shock proteins, as well as the three proteins with higher molecular weights. As seen in lanes 3–6, translation of RNA selected by filter hybridization to each of the four small subclones yields different low molecular weight heat shock proteins, identifying the coding sequences for hsp 28, hsp 26, hsp 23, and hsp 22 with the regions homologous to RNA in subclones 88.1, 88.3, 88.2, and 88.4, respectively. Two-dimensional gel electrophoresis of hybrid-arrested translation mixtures (17, 18) confirms these results and shows that the sequences cloned in λ88 account for all observed low molecular weight heat shock proteins.

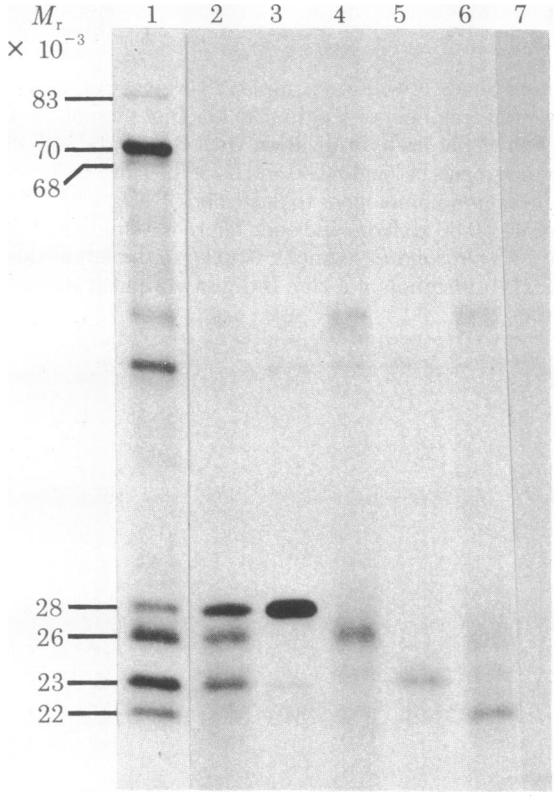

FIG. 4. Cell-free translation of heat shock RNA selected by hybridization to λ88 subclones. Lane 1 of the autoradiogram shows the electrophoretic distribution of [^{35}S]methionine-labeled proteins translated from unselected whole cell heat shock RNA (the dark band at about 40,000 M_r is actin); lane 2, translation products of whole cell heat shock RNA selected by hybridization with 88.1 at 50°C; lanes 3, 4, 5, and 6, translation products of whole cell heat shock RNA selected with 88.1 at 56°C and 88.3, 88.2, and 88.4 at 50°C, respectively; lane 7, translation products of heat shock RNA hybridized to pBR322. Electrophoresis was done in a 10% polyacrylamide/sodium dodecyl sulfate gel.

The protein applied to lane 3 of Fig. 4 was translated from RNA hybridized to the hsp 28 subclone 88.1 at 56°C in 50% (vol/vol)/0.4 M NaCl, while the other four hybridizations were done at 50°C. As seen in lane 2, when message selection is done at the lower stringency and enough protein is analyzed to show an intense band of hsp 28, then hsp 26 and hsp 23 are also found. This evidence for homology between 88.1 and the messages for hsp 26 and hsp 23 is in accord with Southern transfer analysis, which shows partial homology among the *Drosophila* segments of 88.1, 88.2, and 88.3, but not 88.4. Neither translation experiments nor Southern transfer analysis indicate any homology, however, between the four small λ88 subclones or their corresponding messages and the cloned coding sequences for the three large heat shock proteins, hsp 68, hsp 70, and hsp 83.

The sizes of the mRNAs for the four small heat shock proteins were determined from their electrophoretic mobilities relative to DNA standards in an agarose gel containing 10 mM methylmercuric hydroxide, as depicted in Fig. 5. The estimated sizes of the messenger RNAs for hsp 28, hsp 26, hsp 23, and hsp 22 are 1.25, 1.05, 0.96, and 1.03 kb, respectively. Each value is slightly greater than the values found for the corresponding regions of DNA·RNA homology in λ88. Similar experiments with whole cell RNA from embryos not exposed to elevated temperature verify that the accumulation of RNA homologous to λ88 is strongly induced by heat shock.

Evidence that the haploid *D. melanogaster* genome contains only a single copy of each of the heat shock genes cloned in λ88 is provided by comparison of various restriction digests of λ88 with digests of embryo DNA. Such analyses were carried out with *Ava* I, *Bam*HI, *Bgl* I, *Eco*R1, *Pst*, and *Pvu* II. In each case, Southern transfer analysis of the digested embryo DNA probed with ^{32}P-labeled λ88 showed only fragments with sizes equal

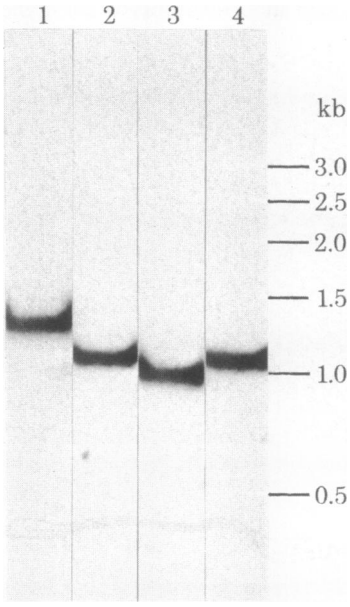

FIG. 5. Size determination of heat shock mRNA. Whole cell heat shock RNA was electrophoresed in a 1.5% agarose gel containing methylmercuric hydroxide, transferred to diazobenzyloxymethyl paper, and hybridized with ^{32}P-labeled DNA from 88.1 (lane 1), 88.3 (lane 2), 88.2 (lane 3), and 88.4 (lane 4). The gel was calibrated with restriction fragments of plasmid pBR322 by using sizes determined by nucleotide sequencing (19).

to the corresponding internal segments of the *Drosophila* DNA of λ88, plus the expected end fragments. Thus, there is only one copy per haploid genome of the genes for each of the four small heat shock proteins.

The direction of transcription of each subcloned heat shock gene was determined by identifying the 3′ terminus of a restriction cleavage site in the DNA chain complementary to heat shock mRNA, as described by Holmgren *et al.* (2). The results, depicted in Fig. 2, were confirmed by similar experiments labeling the 5′termini with polynucleotide kinase.

DISCUSSION

Together with earlier work, the present results show that there are wide differences in the organization of the heat shock loci of *D. melanogaster*. Two of the major heat shock proteins, hsp 68 and hsp 83, are coded by unique sequences at separate loci, 95D and 63BC, respectively (2). The most abundant protein, hsp 70, is coded by multiple genes, two at 87A and three at 87C, with possible variations in other lines of the species (2, 3).

Yet another arrangement is found at 67B: four different genes at the same locus. Although clustered within an interval of 12 kb and activated by the same stimuli, the four genes do not form a common transcription unit. They are oriented in different directions and each gene specifies a separate mRNA.

Intervening sequences appear to be absent from all four of the transcription units cloned from 67B, as shown by the production of only a single S1-resistant DNA·RNA hybrid by each of the four subclones, by the absence of DNA loops in electron micrographs of the hybrids, and by the observation that the mapped transcription units are all somewhat smaller than the sizes estimated for the corresponding messenger RNAs. Although the genes for hsp 68 and hsp 70 also appear to lack intervening sequences, this is not a feature of all heat shock genes, because that for hsp 83 contains an intervening sequence 1 kb long (unpublished data).

At the limited level of sensitivity provided by Southern transfer analysis and message selection, some homology is indicated among three of the segments subcloned from λ88. The significance of this homology is unclear. It is not shared with segments cloned from heat shock loci at 63BC, 87C, or 95D. It may be noted, however, that the mRNA sequences for hsp 70 and hsp 68, located at separate loci, are homologous along most or all of their length (2).

Including the present results, genes for all seven of the major heat shock proteins of *D. melanogaster* have been cloned. A major question now to be addressed is whether these coordinately activated and deactivated genes share common control elements and, if so, what is the structure and organization of such elements at the various loci.

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