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Genetics

Genes for three *Drosophila* heat-shock-induced proteins at a single locus

(gene linkage)

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ABSTRACT **Two plasmids containing cDNA inserts complementary to the messenger RNAs for the 26,000 and 23,000 molecular weight heat-shock-induced proteins from *Drosophila melanogaster* were identified. Both of these probes hybridized to a site of a major heat-shock-induced puff, 67B. Hybridization by the gel transfer technique with genome DNA that had been cleaved by a restriction endonuclease indicated that the two genes are closely linked. This expectation was validated through isolation of a DNA fragment generated by *Bam*HI endonuclease containing genes for the 26,000 and 23,000 molecular weight heat-shock-induced proteins and also a gene encoding the 27,000 molecular weight heat-shock-induced protein.**

Several genes are activated in *Drosophila* larvae or cultured cells as a result of brief exposure to elevated temperature or administration of a variety of drugs (1). This response offers many attractive features as a eukaryotic inducible gene system with apparent coordinate regulation among unlinked loci. Furthermore, the response may be elicited in a cell-free system (2). Gene activation was initially revealed by the appearance of about eight puffs in the polytene chromosomes (3) and later was shown to result in the synthesis of seven or more new proteins (4). Genes for several of the proteins have been assigned to specific loci responsible for the puffs. We wish to report that three of the smaller heat-shock-inducible proteins are encoded at a single puff site 67B. These findings are discussed in terms of the relationship between genes and chromosomes.

MATERIALS AND METHODS

Purification of Nucleic Acids. Chromosomal DNA from Oregon R embryos and plasmid DNAs were purified by conventional means. RNA was purified from the polysomes of heat-treated Kc tissue culture cells as described (5).

***In Vitro* Translation.** The mRNA-dependent rabbit reticulocyte system of Pelham and Jackson (6) was used. RNA isolated from polysomes of heat-shocked Kc cells was hybridized with restriction-endonuclease-cleaved DNA; the DNA was denatured and RNA was added and hybridized as described (5). The DNA·RNA hybrids were separated from unhybridized RNA on hydroxylapatite by the procedure of Lewis *et al.* (7).

Hybridization Analysis. Giant larvae, resulting from a cross of strains Oregon R 236, g-1(gtw^4 homozygous) and g-XII($y^{sc}gt^{xii}$/FM6), were used for squashing. Hybridization of complementary ^{3}H-labeled RNA synthesized by using *Escherichia coli* RNA polymerase (Miles) was done as described (5).

Filter hybridization experiments were carried out with DNA transferred to nitrocellulose from agarose gels as described (5). Hybridizations were carried out in 50% (wt/vol) formamide, 0.75 M NaCl/0.075 M sodium citrate, 0.1% $NaDodSO_4$, 1 mM EDTA, 10 mM Hepes (pH 6.9), and Denhardt's solution (8) at 37°C for 20 hr. After incubation the filters were washed three times in 0.75 M NaCl/0.075 M sodium citrate/0.2% $NaDodSO_4$ at 65°C for 45 min, followed by a 2-hr wash at room temperature in 0.30 M NaCl/0.030 M sodium citrate. Royal X-Omat film and Lightning-Plus Screens (Kodak) were used.

Restriction Endonuclease Digestion and Labeling of DNA. Cleavage with restriction endonucleases (*Bam*HI and *Hin*dIII from Boehringer Mannheim; *Bgl* II, *Eco*RI, *Hin*cII, *Kpn* I, *Pst* I, *Xho* I, and *Sac* I from BioLabs, Beverly, MA) was performed under the conditions suggested by the supplier. Plasmid DNA was labeled by the nick translation reaction as described by Maniatis *et al.* (9), except that 1 ng of DNase I was added along with DNA polymerase (Boehringer Mannheim).

RESULTS

Identification of cDNA Clones Specific for Small Heat-Shock-Induced Proteins. The point of departure for this study was the construction of recombinant plasmids containing cDNA inserts complementary to the smaller species of heat-shock messenger RNA. The mRNA coding for the four smaller heat-shock proteins, approximately 27,000, 26,000, 23,000, and 22,000 molecular weight, sediments at about 12 S (10). RNA from polysomes of heat-shocked cells was fractionated on a sucrose gradient; those fractions that were shown to contain the mRNAs for the smaller heat-shock proteins by *in vitro* protein-synthesis assays were collected and used as a template to synthesize cDNA. The second strand of the cDNA was then synthesized and inserted into plasmid pBR322 as described (5). Two plasmids designated pHS227 and pHS229, having inserts of 320 and 260 base pairs, respectively, showed no base sequence homology in a crossannealing test and hybridized to heat-shock mRNA. In order to determine which of the heat-shock-inducible proteins they represented, each was hybridized with heat-shock mRNA and the hybridized RNA was recovered and translated in a reticulocyte cell-free system. Fig. 1 illustrates the result of this experiment. pHS227 DNA hybridized to mRNA that translated into the 26,000 M_r protein (lane 3), whereas pHS229 DNA hybridized to mRNA that translated into the 23,000 M_r protein (lane 6). The translation products of RNA that failed to hybridize to pHS227 and pHS229 DNAs are shown in lanes 2 and 5, respectively. Shown in lane 4 are the translation products from an aliquot of the same RNA prepa-

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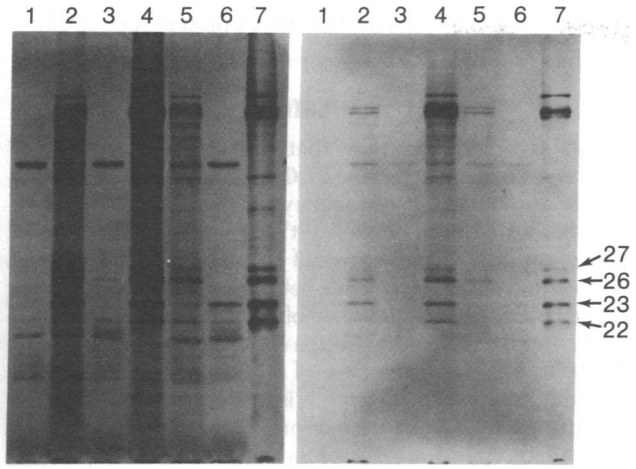

FIG. 1. Cell-free translation of mRNA species selected by hybridization to pHS227 and pHS229 DNA. RNA isolated from polysomes of heat-shocked Kc cells was hybridized with DNA of the pHS227 and pHS229 recombinant DNA clones. Lane 1, no RNA added; lane 2, translation products of mRNA that failed to hybridize to pHS227 DNA; lane 3, translation products of mRNA that hybridized to pHS227 DNA; lane 4, translation products of mRNA not subjected to hybridization; lane 5, translation products of mRNA that failed to hybridize to pHS229 DNA; lane 6, translation products of mRNA that hybridized to pHS229 DNA; lane 7, heat-shock-induced proteins from salivary glands. Arrows indicate the positions of the small heat-shock proteins ($M_r \times 10^{-3}$ is shown) [nomenclature of Ashburner and Bonner (1)]. Shown are 200-hr (*Left*) and 30-hr (*Right*) exposures of the same gel.

ration that had not been subjected to the mRNA selection procedure. The amount of input RNA was one-half of that translated and displayed in lanes 2, 3, 5, and 6. It is obvious that a large proportion of the translation potential of the RNA was lost during the experimental manipulations. Heat-shock-induced proteins from salivary glands are shown in lane 7 as reference standards. By comparing the products of a cell-free translation reaction to which no RNA had been added (lane 1), it can be seen that all bands in lanes 3 and 6 other than the 26,000 and 23,000 M_r heat-shock proteins were due to an endogenous background in the lysate.

pHS227 and pHS229 DNA Sequences Are Linked at Cytological Level. Having shown that DNA from each of these two clones hybridizes to a different heat-shock mRNA, we used *in situ* hybridization to determine the cytological location of these two heat-shock genes. As shown in Fig. 2, sequences from each clone hybridized to the same locus, 67B, the site of a major heat-shock puff. No significant hybridization was detected at other loci in either case.

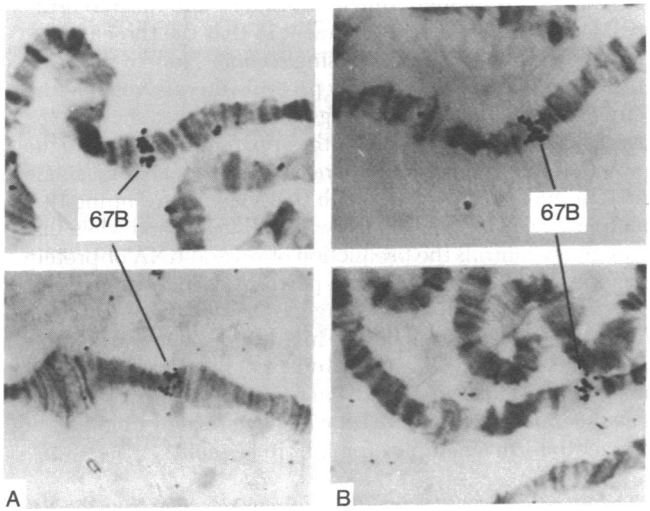

FIG. 2. *In situ* hybridization of pHS227 and pHS229 DNAs to polytene chromosomes. [^{3}H]RNA was synthesized with these two DNAs as template and hybridized to chromosomes; 4×10^5 cpm per slide, 63 days exposure. (*A*) pHS227 cRNA; (*B*) pHS229 cRNA.

pHS227 and pHS229 DNA Sequences Are Linked at Molecular Level. The results of *in situ* hybridization indicate only that the two genes are closely linked in cytological terms. Because of the limited resolution of this technique and the fact that an average band or chromomere contains about 28 kilobases of DNA, these data cannot be interpreted as evidence for close linkage in molecular terms. To approach this question, we resorted to the gel transfer hybridization technique of Southern (11). *Drosophila* DNA was cleaved with nine different restriction endonucleases, fractionated on an agarose gel, and transferred to nitrocellulose filters. Parallel strips of these filters were then hybridized with ^{32}P-labeled nick translated pHS227 and pHS229 probes in order to compare the sizes of genome fragments containing the cloned DNA sequences. Autoradiographs illustrating the results obtained are presented in Fig. 3. The results fall into two categories. Cleavage with five of the endonucleases, *Bam*HI, *Bgl* II, *Hin*dIII, *Kpn* I, and *Xho* I, yielded homologous fragments which appeared to be of identical size for the two probes. These results are most readily interpreted as evidence for the presence of both genes on a single DNA fragment of 10–20 kilobases, depending upon the enzyme in question. The possibility that all five hexamer-recognizing endonucleases yielded fragments of similar size by chance is clearly remote. On the other hand, four restriction endonucleases, *Eco*RI, *Hin*cII, *Pst* I, and *Sac* I, did yield fragments of

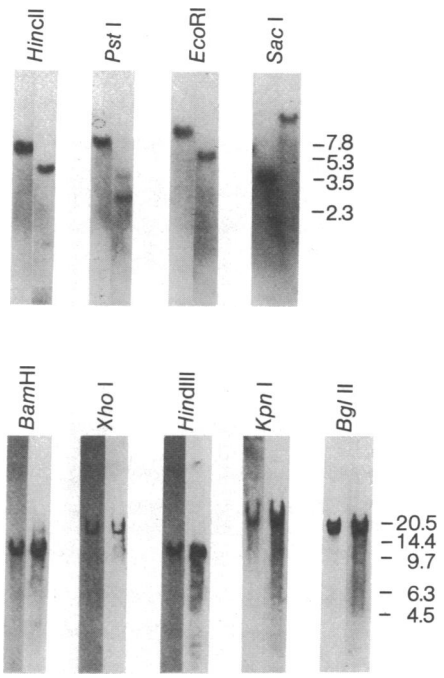

FIG. 3. Size of genome DNA fragments containing sequences homologous to pHS227 and pHS229 DNAs. Three micrograms of *Drosophila* DNA was cleaved with restriction endonucleases *Bam*HI, *Bgl* II, *Eco*RI, *Hin*cII, *Hin*dIII, *Kpn* I, *Pst* I, *Sac* I, and *Xho* I and fractionated by agarose gel electrophoresis. After transfer to nitrocellulose, parallel lanes were hybridized to probes prepared by nick translation of the inserts of pHS227 (1.2×10^6 cpm) and pHS229 (1.6×10^6 cpm). The autoradiographs shown reveal the size of genome fragments homologous to the two probes. Scale on the right indicates the molecular weight of the marker DNAs (*Hin*dIII-cleaved and *Bam*HI/*Eco*RI-cleaved adenovirus 2) in kilobase pairs.

distinguishable size when hybridized with the two probes. These data imply the presence of recognition sites for these enzymes within or between the two coding sequences. We conclude that the 26,000 and 23,000 M_r heat-shock-inducible proteins are both encoded at 67B and that the two genes are closely linked in molecular terms (i.e., within a few kilobases). To prove that this was the case, we screened a library of *Drosophila* *Bam*HI-generated DNA fragments inserted into pBR322 with probes for pHS227 and pHS229. The same bacterial colony from the parallel screens reacted with each of the probes and was called pJ1. This plasmid contains a single *Drosophila* *Bam*HI-generated DNA fragment of 12 kilobases. Restriction endonuclease mapping analysis has shown that, as predicted from the Southern blot hybridization experiments shown in Fig. 3, there are no *Bam*HI, *Bgl* II, *Hin*dIII, *Kpn* I, or *Xho* I sites between the regions of pJ1 that hybridize with pHS227 and pHS229 DNAs. Also in agreement with our interpretation of the data presented in Fig. 3 is that 4.5 kilobases of DNA separates the 23,000 and 26,000 M_r protein genes. Within this region separating the two genes are *Eco*RI, *Pst* I, and *Hin*cII sites (unpublished data).

Sequences from a Third Heat-Shock Gene Are Contained in pJ1. To firmly establish that pJ1 does indeed contain coding regions for the 26,000 and 23,000 M_r proteins, we repeated the mRNA selection/translation experiment described above with pJ1 DNA. As shown in Fig. 4, mRNA that hybridized to pJ1 DNA (lane 2) directed the synthesis of three heat-shock proteins: the 23,000 M_r protein, also selected by pHS229 DNA; the 26,000 M_r protein, also selected by pHS227 DNA; and the 27,000 M_r protein, not selected by either pHS227 or pHS229 DNA. Shown in lanes 4 and 5 are labeled proteins from heat-shocked or control Kc cells. A portion of pJ1, approximately 1.5 kilobases from the 23,000 M_r protein gene, hybridized to mRNA from heat-shocked cells, a result consistent with the presence of a gene for a third heat-shock protein (unpublished data).

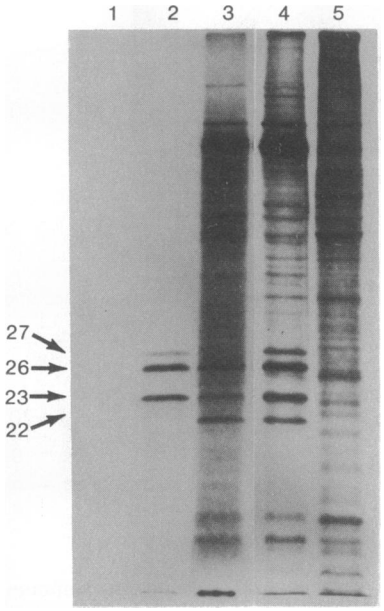

FIG. 4. Cell-free translation of mRNAs selected by hybridization to PJ1 DNA. Lane 1, translation products of mRNA-dependent reticulocyte system without added RNA; lane 2, translation products of heat-shock mRNA that hybridized with pJ1 DNA; lane 3, translation products of RNA that failed to hybridize to pJ1 DNA; lane 4, labeled polypeptides from heat-shocked Kc cells; lane 5, labeled polypeptides from non-heat-shocked Kc cells. Arrows indicate the positions of the 27,000, 26,000, 23,000 and 22,000 M_r heat-shock proteins.

DISCUSSION

The data presented here show that genes encoding three different heat-shock proteins, the 27,000, 26,000, and 23,000 M_r proteins, are located at a single cytological locus, 67B, and, furthermore, are tightly linked at the molecular level. Earlier studies of the heat-shock response of *D. melanogaster* suggested an approximate correspondence between the number of puffs induced, nine, and the number of new proteins that subsequently appear, eight. However, a model in which each locus contains a single gene for one of the proteins is clearly an oversimplification. For example, it is now evident that 87A and 87C are duplicated loci, each harboring several gene copies (1). Furthermore, there appear to be several related proteins ranging from 70,000 to 72,000 in molecular weight encoded at these two loci (5, 12). Data summarized here imply that 67B is also a complex locus. There exist three closely linked regions of transcription accounting for the 27,000, 26,000, and 23,000 M_r proteins. Our results are consistent with those of Peterson *et al.* (13), who mapped the 27,000 and 26,000 M_r species to 67B by using electrophoretic mobility as an index of polymorphism, and McKenzie and Meselson (14), who suggested that the 23,000 or 26,000 M_r protein (or both) is encoded at 67B by *in situ* hybridization experiments.

Let us consider what is known of the structure of the heat-shock genes in terms of the one band–one gene hypothesis (15). According to such a hypothesis, each chromomere with an average DNA content of about 28 kilobases represents a single complementation group and a gene for a single protein. Clearly, even with fragmentary data available concerning only a few genes, several exceptions to that rule are evident. Among these are the 5S RNA genes and the heat-shock genes at 87A and 87C, where several identical or nearly identical genes exist in a tightly linked grouping. The histone genes are another example of tandem repeats, in this case sets of five different genes. Cytological investigations suggest that the 100 or so histone genes (16) and 5S RNA genes (17) are contained within one or two neighboring bands. tRNA gene clusters in *Drosophila* are other examples of tandem repeats of the same gene; in addition, it would appear that tRNA genes for different isoacceptors are sometimes contiguous (18). To this set of exceptions to the single gene–single chromomere rule we must now add the 67B heat-shock locus; this is unique at present in that the three closely linked genes appear to exist as single copies, unlike the cases referred to above. Neither the proteins themselves nor the segments of the genome encoding them have been shown to be homologous. Nevertheless, all of the apparent exceptions to the prediction that each chromomere contains a gene for a single function may be reconciled with that view by assuming that multiple genes may be included within a single band when that set of genes controls the production of several RNA or protein species all of which interact to perform related catalytic or structural functions. The histone genes are one precedent upon which this hypothesis is based. In addition, there are ample precedents for tight linkage of functionally related genes for enzymes in a common pathway in bacteria. Whether the three heat-shock-induced proteins at 67B have related functions or interact to form a complex remains to be seen.

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1. Ashburner, M. & Bonner, J. J. (1979) *Cell* **17**, 241–254.
2. Compton, J. L. & McCarthy, B. J. (1978) *Cell* **14**, 191–201.
3. Ritossa, F. (1962) *Experientia* **18**, 571–573.
4. Tissieres, A., Mitchell, H. K. & Tracy, U. M. (1974) *J. Mol. Biol.* **84**, 389–398.
5. Craig, E. A., McCarthy, B. J. & Wadsworth, S. C. (1979) *Cell* **16**, 575–588.
6. Pelham, H. R. B. & Jackson, R. J. (1976) *Eur. J. Biochem.* **67**, 247–256.
7. Lewis, J. B., Atkins, J. F., Anderson, C. W., Baum, P. R. & Gesteland, R. F. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 1344–1348.
8. Denhardt, D. (1966) *Biochem. Biophys. Res. Commun.* **23**, 641–646.
9. Maniatis, T., Jeffrey, A. & Kleid, D. G. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 1184–1188.
10. Spradling, A., Pardue, M. L. & Penman, S. (1977) *J. Mol. Biol.* **109**, 559–587.
11. Southern, E. M. (1975) *J. Mol. Biol.* **98**, 503–517.
12. Mirault, M. E., Goldschmidt-Clermont, M., Moran, L., Arrigo, A. P. & Tissieres, A. (1978) *Cold Spring Harbor Symp. Quant. Biol.* **42**, 819–827.
13. Peterson, N., Moller, G. & Mitchell, H. (1979) *Genetics* **92**, 891–902.
14. McKenzie, S. L. & Meselson, M. (1977) *J. Mol. Biol.* **117**, 279–283.
15. Judd, B. H. & Young, M. W. (1973) *Cold Spring Harbor Symp. Quant. Biol.* **38**, 573–579.
16. Lifton, R. P., Goldberg, M. L., Karp, R. W. & Hogness, D. S. (1977) *Cold Spring Harbor Symp. Quant. Biol.* **42**, 1047–1052.
17. Wimber, D. E. & Steffensen, D. M. (1970) *Science* **170**, 539–542.
18. Schmidt, O., Mao, J. I., Silverman, S., Hovemann, B. & Soll, D. *Proc. Natl. Acad. Sci. USA* **75**, 4819–4823.