Proc. Natl. Acad. Sci. USA
Vol. 75, No, 11, pp. 5613–5617, November 1978
Genetics

Sequence organization and transcription at two heat shock loci in *Drosophila*

(cloned DNA/hybridization *in situ*/*Drosophila melanogaster*/*Drosophila simulans*)

KENNETH J. LIVAK*, ROBERT FREUND*, MIRIAM SCHWEBER†, PIETER C. WENSINK‡, AND MATTHEW MESELSON*§

* Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138; † College of Basic Studies, Boston University, Boston, Massachusetts 02215; and ‡ Rosenstiel Center, Brandeis University, Waltham, Massachusetts 02154

Contributed by Matthew Meselson, August 14, 1978

ABSTRACT **The heat shock loci of *Drosophila melanogaster* chromosome subdivisions 87A and 87C have been studied by using cloned DNA. Both sites contain a number of copies of a 2.4-kilobase (kb) region homologous to mRNA for the 70,000-dalton heat shock protein. *In situ* hybridization to chromosomal RNA shows that transcripts of this sequence accumulate at both sites after temperature elevation. At 87C there is a 1.5-kb repeated sequence homologous to another heat shock RNA. One cloned segment includes two to three tandem copies of this sequence located 0.8 kb from the beginning of a 2.4-kb message region. RNA complementary to the 1.5-kb repeat accumulates at 87C after temperature elevation, but does not code for any known heat shock protein. In the sibling species *D. simulans*, there are sequences located and transcribed at 87A and 87C that are homologous to the *melanogaster* 2.4-kb message sequence. The 1.5-kb repeat, however, is absent from 87C in *simulans* and no heat shock RNA homologous to it can be detected.**

Transfer of *Drosophila* from room temperature to near 37°C rapidly activates a small number of characteristic genes and greatly reduces the expression of previously active ones. The response was first noted as the induction of puffs at several sites on salivary gland chromosomes, accompanied by regression of preexisting puffs (1, 2). This is paralleled by reduction or cessation of the synthesis of most preexisting proteins and by induction of vigorous synthesis of several characteristic proteins (3). Polysomes isolated shortly after temperature elevation contain newly synthesized RNA complementary to DNA at the heat shock puff sites (4–6). Its translation *in vitro* gives the heat shock proteins (7, 8). The principal protein made at 37°C has a molecular weight of 70,000 and is coded by a 2.6-kilobase (kb) message. Henikoff and Meselson (9) concluded from *in situ* hybridization experiments that this message is homologous to DNA at chromosome subdivisions 87A and 87C, two major heat shock puff sites. They also found that temperature elevation induces the synthesis of additional RNA, homologous to extensive sequences at 87C but not at 87A, as also suggested by Spradling *et al.* (6). Using cloned DNA, we confirm these conclusions and present a more detailed analysis of sequence organization and transcription at 87A and 87C in *D. melanogaster* and its sibling species *D. simulans*.

MATERIALS AND METHODS

RNA and DNA. Polysomal RNA, polysomal poly(A)-containing RNA, and gel-fractionated 2.6-kb mRNA were prepared from cells cultured at 36°C for 60 min or, for whole cell RNA, 9 min (9). RNA labeled *in vivo* was prepared from cells concentrated 20-fold into 128 mM NaCl/47 mM KCl/19 mM $CaCl_2$/$^{32}PO_4$ at 1 mCi/ml (1 Ci = 3.7×10^{10} becquerels) and kept at 36°C for 60 min before harvest. RNA was labeled *in vitro* by partial degradation and treatment with [γ-^{32}P]ATP and polynucleotide kinase (10). Cloning of sheared *melanogaster* embryo DNA in plasmid pMB9 in *Escherichia coli* HB101 and screening by colony hybridization will be described elsewhere (ref. 11; R. Stern, P. Gergen, and P. C. Wensink). Plasmid DNA was isolated by ethidium bromide/CsCl gradient centrifugation of cleared lysates of cells cultured with chloramphenicol (12). Ethidium bromide was removed by isopropanol extraction and dialysis. Work with recombinant plasmids was done under P2 + EK1 conditions. Tritiated cRNA was prepared essentially according to Wensink *et al.* (11) and precipitated with ethanol. Labeled DNA was prepared by 1-hr incubation at 13°C of 10 μg of DNA per ml/10 mM Tris·HCl (pH 7.4)/5 mM NaCl/10 mM $MgCl_2$/10 mM 2-mercaptoethanol/100 μg of gelatin per ml/1.8 μM [^{3}H]- or [^{32}P]dNTPs/25 units of *E. coli* DNA polymerase I per ml/5 ng of DNase I per ml, followed by phenol extraction and Sephadex G-50 chromatography.

Restriction Mapping and Gel Electrophoresis. Maps were deduced from the sizes of fragments produced by single, double, and partial restriction digests analyzed on 1–1.5% agarose gels in TAE buffer (13) and on 5% polyacrylamide gels in TBE buffer (14). *Hin*dIII and *Eco*RI fragments of phage λ (15) and *Hae* III fragments of pMB9 (G. K. Sim, personal communication) were used as size standards. For strand separation, DNA denatured in 0.1 M NaOH/2 mM EDTA was electrophoresed on a 1% agarose gel in TBE. DNA fragments were blotted from agarose gels onto nitrocellulose filters (16) and hybridized to ^{32}P-labeled RNA overnight at 65° in 0.3 M NaCl/0.03 M Na citrate, pH 7/0.5% sodium dodecyl sulfate/20 μg of yeast RNA per ml. Hybridization with ^{32}P-labeled DNA was done according to Botchan *et al.* (17). After six 45-min washes with 3 mM Tris base at room temperature, filters were dried and autoradiographed. Hybridization at 37°C with [^{32}P]poly(A) and washing were done in 0.15 M NaCl/0.015 M Na citrate, pH 7.

Subclones. Subclone 229.1 was made by combining 0.2 μg of pBR 322 DNA (18) and 0.5 μg of 229 DNA, both cleaved by restriction endonucleases *Bam*HI and *Sal* I, in 50 μl of 10 mM Tris·HCl, pH 7.9/50 mM NaCl/10 mM $MgCl_2$/0.2 mM dithiothreitol/1 mM ATP. After addition of phage T4 ligase (gift of Hans Lehrach), the mixture was incubated 3 hr at 13°C, diluted 5-fold with the ligation buffer, kept overnight at 13°C, and used to transform *E. coli* HB101 (19). DNA from cultures of ampicillin-resistant tetracycline-sensitive colonies was restricted to identify a plasmid carrying the desired *Bam-Sal* fragment in the pBR 322 *tet* gene. Subclones 232.1 and 232.2 were constructed by inserting *Pst* fragments of 232 in the *Pst*

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Abbreviations: kb, kilobase; Pipes, 1,4-piperazinediethanesulfonic acid.

§ To whom reprint requests should be addressed.

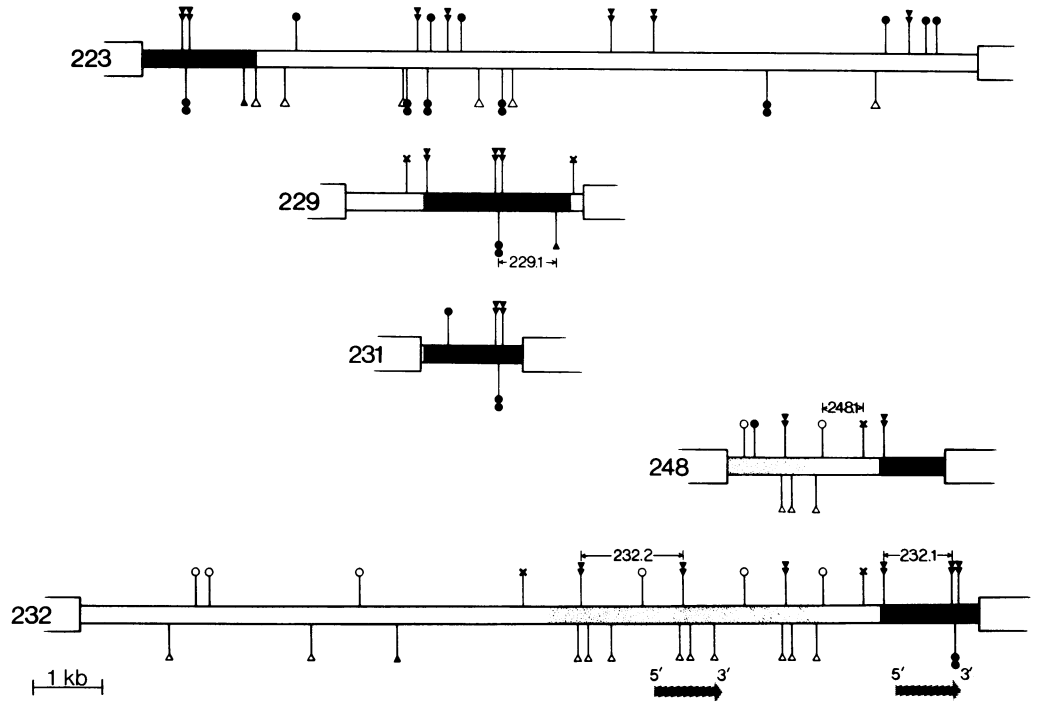

FIG. 1. Restriction maps of cloned DNA segments from *D. melanogaster* heat shock loci 87A and 87C. Cleavage sites are shown for *Bgl* I (●), *Hin*dIII (○), *Pst* (↕), *Xho* (×), Bam (‡), *Hin*cII (△), and *Sal* (▲). *Sal* sites are also *Hin*cII sites. All sites are shown for each enzyme except that 232 was mapped with *Bgl* I only in the region homologous to 2.6-kb message, where no site was found. The lengths of the inserts, in kb, are 12.3, 3.3, 1.5, 3.3, and 13.2 for 223, 229, 231, 248, and 232, respectively. Comparison of restriction sites suggests that the left end of 232 overlaps with cDm 702 and 704 of Lis *et al.* (24). Subcloned fragments are indicated as 229.1, 248.1, 232.1, and 232.2. Filled sections depict homology to the 2.6-kb heat shock message, and stippled sections show the 1.5-kb tandem repeat region. The latter is homologous to heat shock RNA different from the 2.6-kb mRNA. Both RNAs hybridize to the same DNA strand, with 5′→3′ polarity as shown. All five clones probably derive from 87A or 87C, the only sites where the subclones from the 2.4-kb message sequence hybridize strongly *in situ*. The only site where both the 1.5-kb repeat and the message subclones hybridize is 87C, hence 232 and 248 derive from that site. It is likely that 223 is from 87A, because it hybridizes there much more strongly than at 87C.

site of pBR 322. Subclone 248.1 contains a *Hin*dIII-*Xho* I double digestion fragment of 248 inserted at the *Hin*dIII and *Sal* I sites of pBR 322.

Hybrid-Arrested Translation. Hybrid-arrested translation was done essentially according to Paterson *et al.* (20). Peptides resulting from translation with nuclease-treated rabbit reticulocyte lysate (20, 21) were electrophoresed on 10% polyacrylamide/sodium dodecyl sulfate gels (22), which were then autofluorographed (23).

Hybridization *In Situ*. Tritium-labeled cRNA (9×10^7 dpm/μg) at about 10 μg/ml was hybridized to chromosomal DNA as previously described (9), using *D. melanogaster* Oregon R, *D. simulans* Guatemala, or the interspecies hybrid, except that 5S RNA was omitted and the hybridization buffer was 0.01 M 1,4-piperazinediethanesulfonic acid (Pipes) (pH 6.8)/0.45 M NaCl/0.045 M Na citrate (pH 7)/50% (wt/wt) formamide (Fluka). For hybridization to chromosomal RNA, squashes were prepared as above, except that larvae were dissected in 45% (vol/vol) acetic acid, ethanol/acetic fixative was not used, and the RNase and alkali treatments were omitted. For 10 slides, 1 μg of ^{3}H-labeled DNA (2×10^7 dpm/μg) was ethanol precipitated with 100 μg of sonicated calf thymus DNA and 100 μg of *E. coli* tRNA, dissolved in 20 μl of H_2O, kept 3 min at 100°C, frozen in liquid N_2 and, just before use, made up to give 10 μg of [^{3}H]DNA per ml, 50% formamide, 0.01 M Pipes (pH 6.8), and 0.4 M NaCl. After hybridization with this solution overnight at 37°C, slides were washed 5 min at 37°C in 40% formamide/0.01 M Pipes (pH 6.8)/0.4 M NaCl and 5 hr at room temperature in 0.3 M NaCl/0.03 M Na citrate (pH 7), dried through ethanol, and autoradiographed.

RESULTS

Isolation and Restriction Mapping of Plasmids. Colonies containing cloned random segments of *D. melanogaster* DNA were screened by hybridization with polysomal poly(A)-containing heat shock RNA labeled *in vitro*. Of 23,000 colonies, 38 were scored as positive and were retested using gel-fractionated 2.6-kb heat shock mRNA labeled *in vivo*. Five clones unambiguously hybridized to the purified mRNA. Their plasmids are designated pPW 223, pPW 229, pPW 231, pPW 232, and pPW 248. Restriction maps of the cloned segments are shown in Fig. 1. Numerous similarities among them are evident. The map of 248 is included in that of 232. There are, however, several differences, suggesting that the five segments represent at least three distinct copies or alleles, a conclusion supported by mapping with *Bgl* II, *Hae* II, and *Hpa* II. A notable feature of 232, shown by the pattern of *Hin*cII, *Hin*dIII, and *Pst* I restriction sites, is a 1.5-kb sequence tandemly repeated twice, with at least part of a third copy. This 1.5-kb sequence corresponds to the αβ element studied by Lis *et al.* (24).

Homologies to Heat Shock RNA. Restriction digests of the plasmids were electrophoresed on agarose gels, blotted onto nitrocellulose paper, and hybridized with gel-purified 2.6-kb message labeled *in vitro*. The regions of homology are shown in Fig. 1. The maximum extent of homology, estimated as the distance between the restriction sites beyond which no hybridization is observed, is 2.4 kb, while the minimum is about 2.1 kb. Within the region of mRNA homology, there are numerous restriction sites common to the five plasmids. The message region of 231, however, differs from that in the other

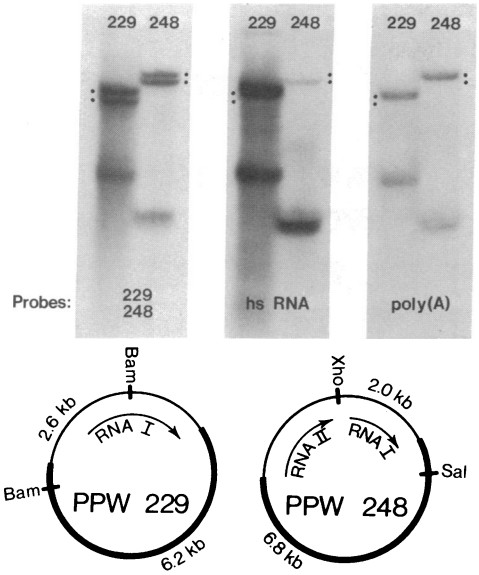

FIG. 2. Orientation of heat shock RNA on plasmids 229 and 248. Plasmids were cleaved at the indicated restriction sites, denatured, electrophoresed, blotted onto nitrocellulose, and hybridized with ^{32}P-labeled 229 and 248 DNA, heat shock (hs) RNA, or poly(A). Dots show the positions of the separate strands of the 6.2-kb 229 fragment and the 6.8-kb 248 fragment. RNA I is the 2.6-kb heat shock message and RNA II is the heat shock RNA homologous to the 1.5-kb repeat.

plasmids by having a *Bgl* I site and lacking a *Pst* site, suggesting that the message sequence is polymorphic.

Whole cell heat shock RNA hybridizes to the 2.4-kb message sequence and also to the 1.5-kb sequence tandemly repeated in 232 and present in 248. Our preparations of polysomal poly(A)-containing RNA also hybridize to the 1.5-kb sequence, but this may be due to contamination by nonpolysomal RNA. The only other hybridization detected with whole cell RNA was a small amount to the 0.8-kb *Hin*dIII–*Pst* fragment between the 1.5-kb repeat and the 2.4-kb message region.

Direction of Transcription. The experiment illustrated in Fig. 2 determines the orientation of cloned DNA with respect to RNA polarity. *Bam* cleaves 229 into fragments of 6.2 kb and 2.6 kb, each containing one of the two dA-dT joints used to construct the plasmid. When denatured with alkali and electrophoresed on a neutral gel, the 6.2-kb fragment separates into two bands. Only the slower hybridizes with ^{32}P-labeled poly(A)-containing polysomal heat shock RNA, showing that this strand is complementary to the 2.6-kb mRNA. Also, only the slower strand hybridizes to [^{32}P]poly(A), showing it contains

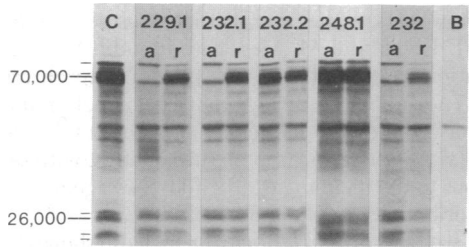

FIG. 3. Hybrid-arrested translation of heat shock RNA. The autoradiogram shows the electrophoretic distribution of [^{35}S]methionine-labeled proteins translated *in vitro* from heat shock polysomal RNA hybridized with the indicated plasmid DNA and either added directly to the translation system (a) or first released from hybrid by denaturation (r). Control translations were done without DNA (C) and with neither RNA nor DNA (B). Lines indicate the positions of heat shock proteins at 83,000, 72,000, 70,000, 68,000, 28,000, 26,000, 23,000, and 22,000 daltons.

the poly(dT) of the dA-dT joint. Considering that 229 was constructed with poly(dA) tails on the 3′ ends of the *D. melanogaster* DNA and poly(dT) tails on pMB9, we deduce the orientation of the 2.4-kb message sequence shown in Fig. 1. The same logic was used to determine the orientation of the 1.5-kb repeat in 248.

Subclones. As indicated in Fig. 1, subclone 229.1 contains the 0.84-kb *Bam–Sal* fragment homologous to the 3′ end of the 2.6-kb message. Subclone 232.1 contains the 1.0-kb *Pst* fragment homologous to the 5′ end of the message. These are termed the 3′ and 5′ segments, respectively. A copy of the repeated 1.5-kb *Pst* fragment is subcloned as 232.2 and is termed the 1.5-kb repeat segment. The intervening 0.6-kb *Hin*dIII–*Xho* fragment is subcloned as 248.1 and is termed the interval segment.

Hybrid-Arrested Translation. Plasmid DNA from the subclones was tested by hybrid-arrested translation in order to determine which DNA segments code for heat shock proteins. Hybridization with DNA containing a coding sequence blocks *in vitro* translation of the complementary message (20). As may be seen in Fig. 3, translation with no DNA added shows bands corresponding to the eight heat shock proteins consistently observed on sodium dodecyl sulfate electrophoretic gels (7, 8). The other bands can be accounted for by residual synthesis of proteins not induced by heat shock, with the exception of a band at 38,000 daltons that in some experiments is induced by heat shock. Hybridization with plasmid DNA containing either the 3′ or 5′ segment specifically blocks the synthesis of the 70,000-dalton protein. The simultaneous elimination of the 72,000-dalton protein shows that it is specified by a different but homologous RNA sequence, or that there is a difference in translation or subsequent modification. Heat shock mRNA hybridized to the 3′-segment directs the synthesis of protein in the 40,000-dalton region not seen in the control, as expected if translation is arrested about halfway along the message. This confirms the orientation of the message region deduced above. DNA from subclones containing the 1.5-kb repeat segment and the interval segment has no observed effect on the translation of heat shock RNA. Thus, neither of these sequences appears to code for any of the known heat shock proteins or for any other protein seen on our gels. This is confirmed by the observation that the gel pattern for arrest by 232 itself is identical to that for the message subclone 232.1.

***In Situ* Hybridization to DNA.** As shown in Fig. 4*a*, the 3′ segment subcloned in 229.1 hybridizes strongly at 87A and 87C, as does the 5′ segment subcloned in 232.1. After long exposure using nick-translated DNA as probe, both subclones label two additional sites, 87D and 95D. The latter results from partial homology between the 2.4-kb message sequence and the coding sequence for the 68,000-dalton heat shock protein that is specified at 95D (R. Holmgren, R. Morimoto, R. Freund, K. J. Livak, and M. Meselson, unpublished data). As shown in Fig. 4*b*, the 1.5-kb repeat segment subcloned in 232.2 hybridizes principally at 87C and, less strongly, at the most proximal division of every chromosome arm except 3R and at 10C, 42B, and 102DE, but not at 87A. The interval segment subcloned in 248.1 hybridizes strongly at 87C and weakly at 87A. In the sibling species *D. simulans*, heat shock puffs occur at the same sites as in *D. melanogaster* (2), and the 3′ and 5′ segments hybridize strongly at 87A and 87C. In contrast, the 1.5-kb repeat segment does not show hybridization in *simulans* at 87C or at any other location except the proximal divisions labeled in *melanogaster*. The lack of homology to 232.2 at *simulans* 87C is nicely shown in Figure 4*c* by the one-sided labeling of the synapsed chromosomes of a *melanogaster–simulans* hybrid. When the 1.5-kb repeat segment is hybridized to nuclei of both species on the same slide, the number of autoradiographic

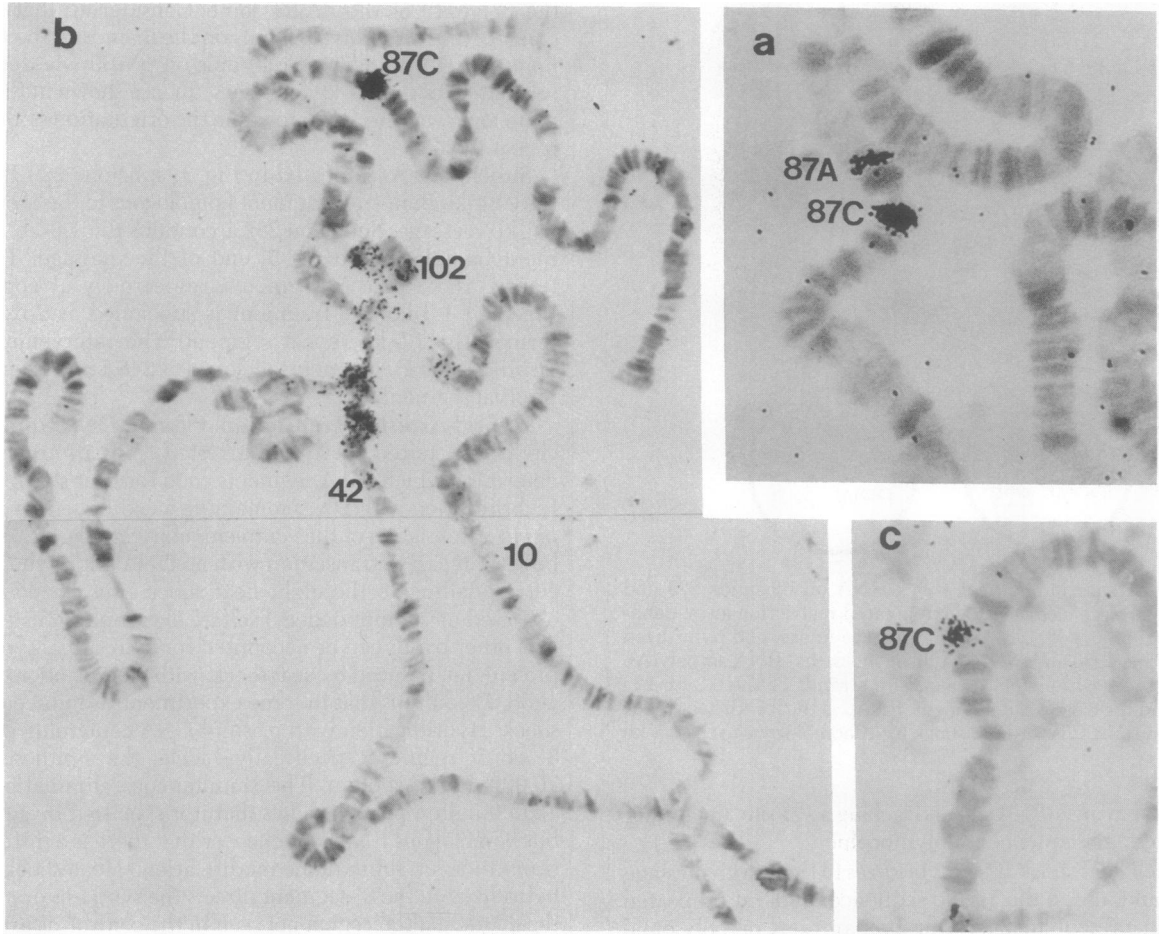

FIG. 4. Hybridization *in situ* by copy RNA from plasmids 229.1 and 232.2 to chromosomal DNA. Polytene chromosomes were hybridized with [^{3}H]RNA copied from plasmid DNA and autoradiographed. (*a*) *D. melanogaster* hybridized with 229.1. (×1260.) (*b*) *D. melanogaster* hybridized with 232.2. (×725.) (*c*) Chromosomes from a *melanogaster–simulans* hybrid larva hybridized with 232.2. (×1100.) Exposures were for 54, 12, and 37 days, respectively. Grains are observed at the indicated sites and, for 232.2, in chromocentral regions.

grains at *melanogaster* 87C is hundreds of times greater than the background number at *simulans* 87C or at 87A of either species. Because the 1.5-kb repeat is homologous to no more than about 40 kb of DNA at 87C (24), we conclude that it is not present at 87C in *simulans* or at 87A in either species.

Transcriptionally Active Sites. In order to distinguish the sites at which transcription occurs, subcloned DNA probes were hybridized *in situ* to chromosomal RNA, rather than DNA. This is achieved by omitting the alkali denaturation and RNase digestion steps used in conventional hybridization *in situ* (25). Hybridizations were done with larvae kept at 36°C for 3 min and 15 min and with untreated larvae. Representative results are shown in Fig. 5. Hybridization with either the 3′ or 5′ segments shows that transcripts of the message sequences accumulate in both *melanogaster* and *simulans* at 87A and 87C but not elsewhere. In contrast, RNA complementary to the 1.5-kb repeat segment accumulates in *melanogaster* only at 87C and not at all in *simulans*. In *melanogaster*, the interval segment hybridizes to RNA at 87C and, less strongly, at 87A, while in *simulans* weak hybridization was found equally at both sites. For all four probes, no grains are found at any site without heat shock, and 3 min after heat shock there are nearly as many grains as at 15 min.

DISCUSSION

Earlier studies indicated the existence at 87A and 87C in *D. melanogaster* of sequences homologous to a 2.6-kb polysomal poly(A)-containing heat shock RNA that codes for the 70,000-dalton heat shock protein (6–9, 26). The present findings confirm this picture. The 2.6-kb message is homologous to a 2.4-kb sequence, internal subclones of which hybridize at both sites. Also, hybridization of heat shock RNA with these subclones specifically blocks translation *in vitro* of the 70,000-dalton heat shock protein. Essentially the same findings have recently been reported by Schedl *et al.* (27). The presence of at least two copies of the message sequence at 87A and at least three at 87C, consistent with the differences found in the maps of our five plasmids, has been shown by restriction analysis of DNA from wild-type flies and flies lacking the 87C heat shock locus (K. J. Livak and M. Meselson, unpublished data). The hybridization *in situ* of message subclones to chromosomal RNA at 87A and 87C indicates that heat shock induces transcription of the message sequences at both sites. In the sibling species *D. simulans*, the same picture obtains, with sequences homologous to the *melanogaster* 2.4-kb message region located and transcribed at *simulans* 87A and 87C.

Earlier studies with *melanogaster* had also shown that there is heat shock RNA that hybridizes to extensive sequences at 87C but not at 87A, even in the presence of a large excess of competing 2.6-kb message (9). In the present study we find a 1.5-kb tandemly repeated sequence at *melanogaster* 87C that is homologous to RNA which accumulates exclusively at this site after temperature elevation. The homology of most of the noncompetable RNA to the 1.5-kb repeat is shown by the observation that a mixture of 2.6-kb message and RNA copied *in*

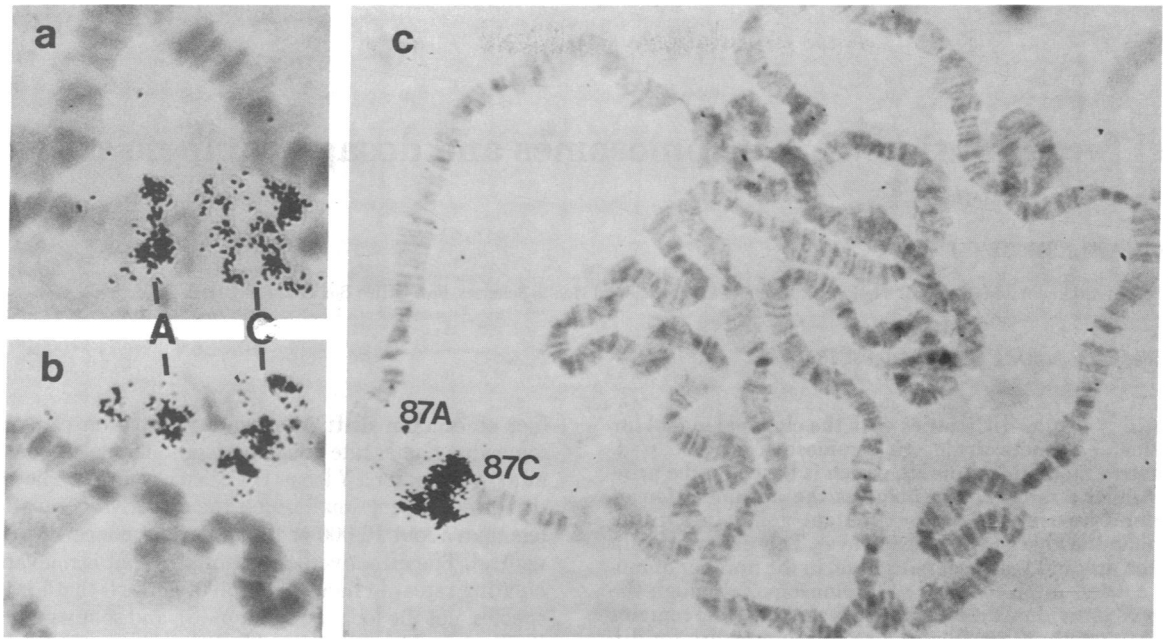

FIG. 5. Hybridization *in situ* by DNA from plasmids 229.1 and 232.2 to chromosomal RNA after heat shock. Polytene chromosomes from larvae kept at 36°C for 15 min were hybridized with plasmid [^{3}H]DNA under conditions favoring hybridization to chromosomal RNA and autoradiographed. (*a*) *D. melanogaster* and (*b*) *D. simulans* hybridized with DNA from message subclone 229.1; (*c*) *D. melanogaster* hybridized with DNA from the 1.5-kb repeat subclone 232.2. Magnifications ×1175, ×1175, and ×725, respectively. Exposure was for 17 days. Grains are found only at the puffs at 87A and 87C for 229.1 and at 87C for 232.2.

vitro from the cloned repeat competes away nearly all hybridization by heat shock RNA at 87C (S. Henikoff, unpublished data). An analogous situation may exist in *D. hydeii*, in which the heat shock locus at 2-48BC hybridizes with two classes of RNA, only one of which is thought to be message (28). Because the 2.4-kb message sequence and the 1.5-kb repeat represented on 232 are oriented in the same direction with respect to RNA polarity, they could belong to the same transcription unit, a possibility consistent with the accumulation at 87C of heat shock RNA homologous to the interval segment subcloned in 248.1. This subclone, unlike 232.2, has homology to RNA that accumulates in response to heat shock at both 87A and 87C in *melanogaster* and in *simulans*. The *Drosophila* sequences cloned in 232.2 and 248.1 appear not to code for protein because they are not homologous to the 2.6-kb message and are not found to affect *in vitro* translation when hybridized to heat shock RNA.

Our finding that the 1.5-kb repeat does not code for any known heat shock protein accords with the suggestion that its transcripts may perform a regulatory function (9). However, in *simulans*, the closest known relative to *melanogaster*, the 1.5-kb repeat sequence is absent from 87C and no transcripts of it are found at any site. This may mean that the 1.5-kb sequence has no function or that it performs a function missing in *simulans* but possibly of selective value to *melanogaster*. Alternatively, a quite different sequence may serve an analogous role in *simulans*. Whether or not the 1.5-kb sequence performs an adaptive function, its evolutionarily rapid introduction at 87C in *melanogaster* or its removal from that site in *simulans* may reflect a general process for producing diversity.

We are grateful to Pamela Dunsmuir for assistance with hybridization *in situ* to chromosomal RNA, to Ralph Stern for assistance in screening clones, and to the National Institutes of Health and the National Science Foundation for support.

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