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Cloning of heat-shock locus 93D from *Drosophila melanogaster*

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Using the microcloning approach a number of recombinant λ phages carrying DNA from the 93D region have been isolated. Screening genomic libraries, cloned in phage λ or cosmid vectors, with this isolated DNA yielded a series of overlapping DNA fragments from the region 93D6-7 as shown by *in situ* hybridization to polytene chromosomes. *In vitro* ^{32}P-labelled nuclear RNA prepared from heat-shocked third instar larvae hybridized specifically to one fragment within 85 kb of cloned DNA. The region which is specifically transcribed after heat shock could be defined to a cluster of internally-repetitive DNA and its neighbouring proximal sequences. Over a sequence of 10–12 kb in length the DNA is cut into repeat units of ~280 nucleotides by the restriction endonuclease *Taq*I. The *Taq*I repeat sequences are unique in the *Drosophila* genome.

Key words: heat shock/*Drosophila melanogaster*

Introduction

All living cells from bacteria to man seem to respond in a similar fashion to a rise of several degrees in temperature. After heat shock most RNA and protein synthesis is reduced and synthesis of a few species of heat-shock RNAs commences; most of these RNAs are preferentially translated (for review, see Ashburner and Bonner, 1979; Schlesinger *et al.*, 1982). In *Drosophila*, polytene tissues like the salivary glands allow the identification of heat-induced gene loci through the formation of puffs. After heat shock six major puffs are activated at the loci 63B, 67B, 87A, 87C, 93D and 95D. All these heat-shock loci have been cloned and analyzed in detail except 93D.

The RNA transcribed at heat-shock locus 93D seems to be mostly nuclear and devoid of protein coding capacity. It can be identified both in the poly A$^+$ as well as the poly A$^-$ fraction of nuclear RNA, part of this transcript is also found in the poly A$^-$ RNA fraction of the cytoplasm (Lengyel *et al.*, 1980). Heat-shock locus 93D can be induced by benzamide independently of all the other heat-shock loci. Recent progress in the elucidation of the function of its transcript in the nucleus (Dangli *et al.* 1983) prompted us to clone the heat-shock gene 93D. The cloned gene may allow us not only to analyze the assembly of the giant RNP structure found at this locus after induction but also to study its special role within the heat-shock response.

Results

Cloning of genomic DNA from heat-shock puff 93D

To enter the chromosomal region 93D we made use of the microcloning technique described by Scalenghe *et al.* (1981). Starting from ten heat-induced puffs cut out of a salivary gland squashed preparation and carried through the microcloning procedure 175 cloned *Eco*RI fragments were recovered (Hovemann *et al.*, unpublished data). As a guide for the identification of heat-shock gene locus 93D, defined as the overlapping region of the deficiency mutants Df(3R)eGP4 and Df(3R)GC14 at the cytogenetic locus 93D6-7 (Mohler and Pardue, 1982), we performed a differential Southern screening with twenty different clones resulting from the microcloning experiment (Figure 1). A clone show-

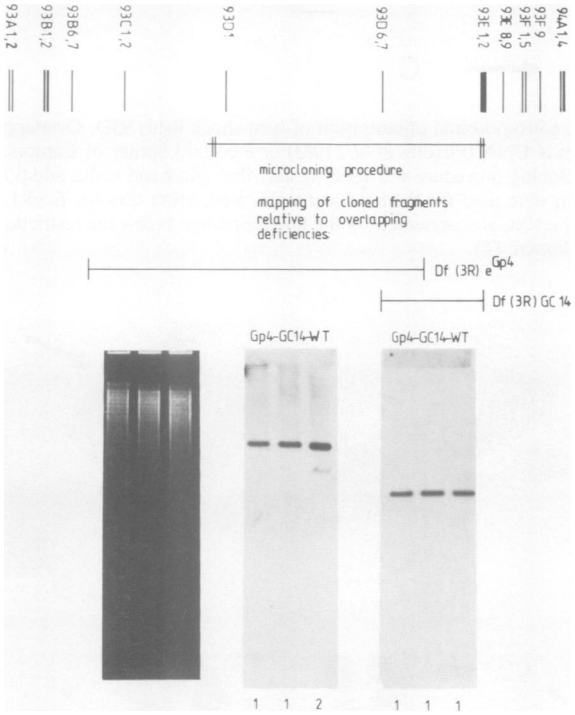

Fig. 1. Identification of the chromosomal region 93D 6-7. On top a schematic drawing of the cytogenetic map of polytene chromosomes from position 93A to 94A is shown. The puffed region microdissected and microcloned into phage lambda NM 641 is indicated by the bar below the map. The amount of DNA missing in one chromosome of the deficiency mutants Df(3R)eGP4 and Df(3R)GC14 respectively is represented by the two lines in the middle. Two examples of a Southern hybridization experiment are shown: to the right equal signal intensity for hybridization to DNA from eGP4, GC14 and wt DNA is indicative for DNA deriving from a region outside of both deficiencies (Hovemann *et al.*, unpublished data). In the middle an example for the signal ratio 1:1:2 of DNA deriving from the overlapping part of the deficiencies is shown, hybridization probe: clone 4/6. For all experiments equal amounts of eGP4, GC14 and wt DNA, digested with *Eco*RI, have been loaded as shown by the ethidium bromide staining pattern of a corresponding agarose gel.

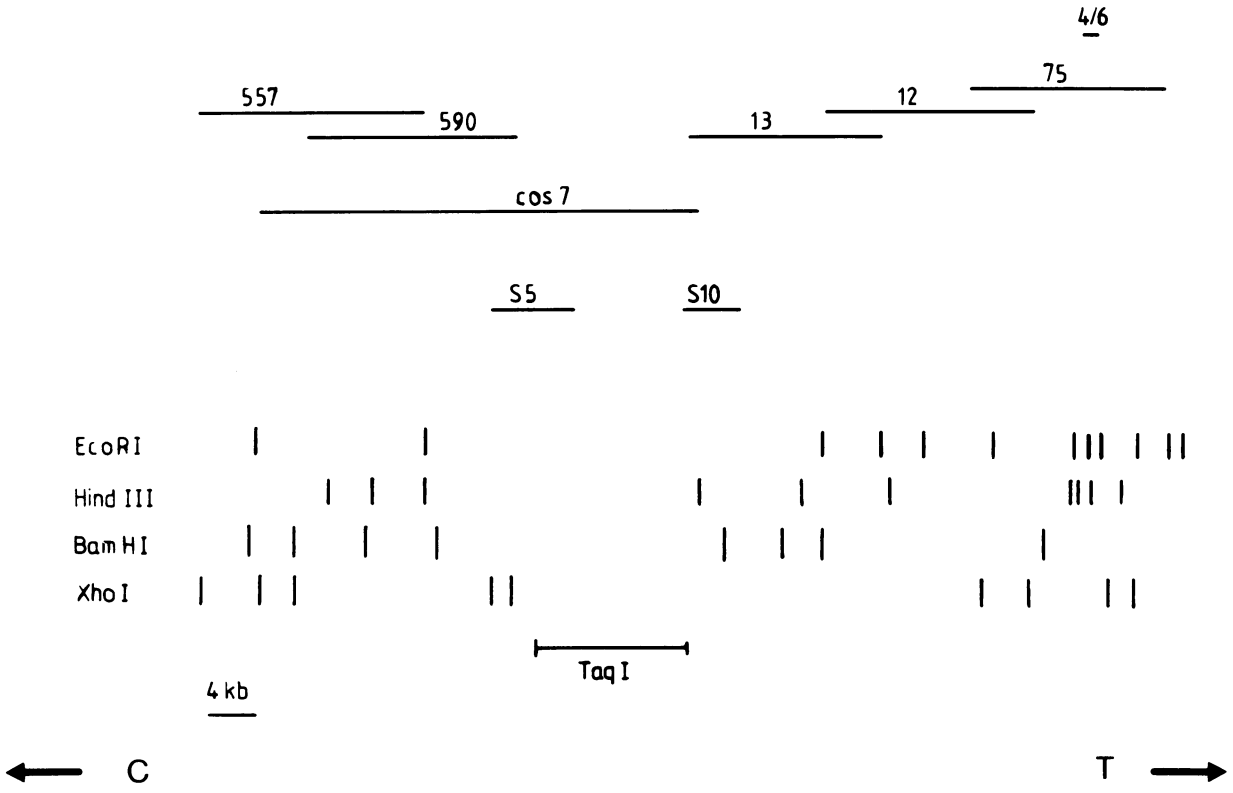

Fig. 2. Chromosomal organization of heat-shock locus 93D. Overlapping DNA fragments from heat-shock locus 93D isolated either from a lambda library of Oregon R DNA (Pirrotta *et al.*, 1983) or a cosmid library of Canton S DNA are indicated as horizontal lines. Fragment 4/6 deriving from the original microcloning procedure was used to start the right-hand walk, additional 'microclones' deriving from the region of the eGp4 deletion but outside of the GC14 deletion were used for the left-hand walk. Restriction sites for *Eco*RI, *Hind*III, *Bam*HI and *Xho*I are shown as vertical bars. The *Taq*I repeat region, referred to in the text, is represented by the horizontal line below the restriction map. Arrows indicate the orientation of the cloned DNA relative to centromere (C) and telomere (T).

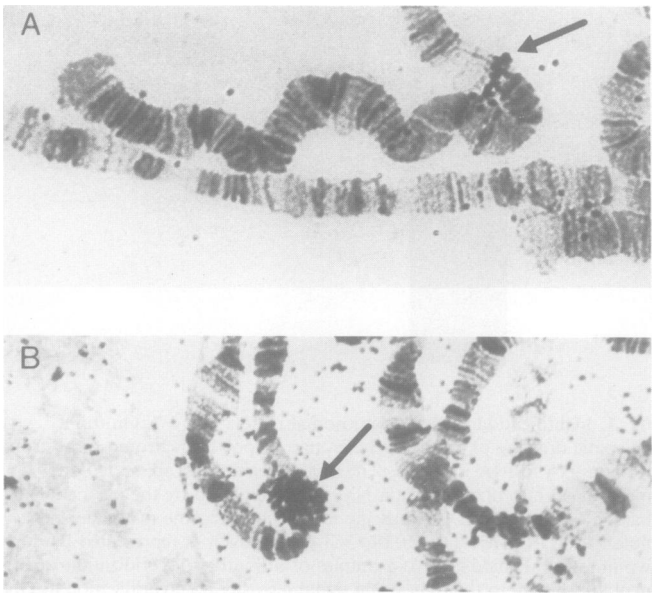

Fig. 3. *In situ* hybridization of cos 7 DNA. ^{3}H-labelled cos 7 DNA was hybridized *in situ* to squashed preparations of polytene chromosomes from Oregon R third instar larvae. **(A)** Hybridization to chromosomal DNA. **(B)** Hybridization to chromosomal RNA. Sp. act. was 1 x 10^7 d.p.m./μg; exposure time: 3 days.

ing half the hybridization intensity on Southern blots of the heterozygous eGp4 and GC14 DNA compared with wt-DNA (number 4/6 in Figure 2) was used to screen a phage λ library of overlapping genomic DNA fragments (kindly provided by V. Pirrotta). In this way we were able to start a chromosomal walk from a region very close to the heat-shock locus 93D. Additional microclones have been used for screening the genomic library and thereby saturating the region with overlapping DNA fragments and accelerating the conventional walking procedure. As a test for the heat-shock gene 93D we hybridized *in vitro* labelled nuclear RNA from heat-shocked *Drosophila melanogaster* third instar larvae with Southern blots of the DNA, which was included in the walk. In this way we tested overlapping DNA fragments isolated from the chromosomal region 93D (Figure 2). One piece of ~15 kb of DNA between phages λ 590 and λ 13 left uncloned in the series of cloned overlapping fragments appeared to be missing in the EMBL4 λ library used. Since none of the recombinants showed hybridization to nuclear RNA isolated from heat-shocked third instar larvae, we expected the heat-shock gene within the uncloned gap of ~15 kb in length.

Screening experiments with fragments from the very end of the cloned *Drosophila* DNA in phages λ 590 and λ 13 respectively with different libraries of cloned genomic Oregon R and Canton S DNA including our own unamplified libraries remained fruitless. This result suggests two possible explanations: either the uncloned DNA between λ 590 and λ 13 is

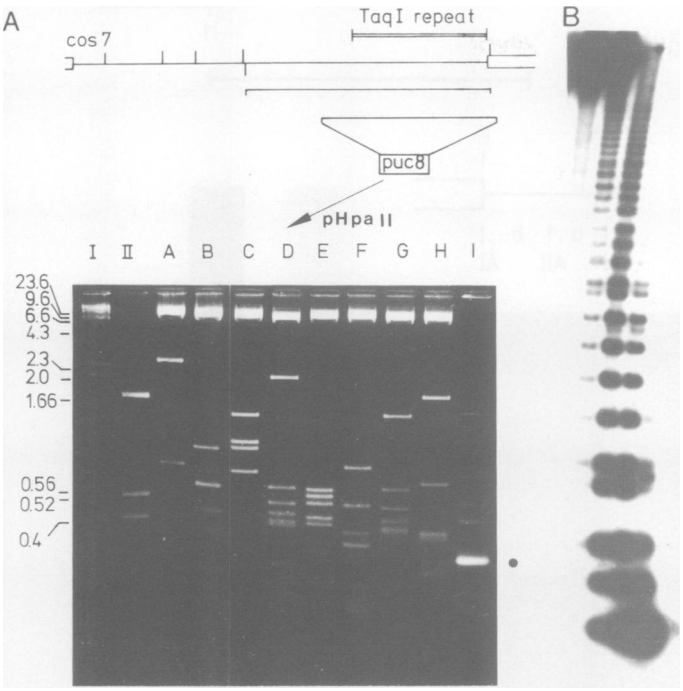

Fig. 4. (A) Restriction analysis of the heat-shock gene region. The restriction map of cos 7 DNA is shown, vertical bars pointing up represent *Hind*III restriction sites, one *Eco*RI restriction site is indicated by the single bar pointing down. Recloned fragments are shown below the cos 7 restriction map. The ethidium bromide staining pattern of an agarose gel electrophoresis contains as size marker wt-lambda DNA, *Hind*III digested in **lane I** and pUC 8 DNA, *Hinf*I digested, in **lane II. Lanes A–I** contain pHpaII DNA digested with: *Eco*RI-*Hind*III, *Sau*3A, *Ava*II, *Dde*I, *Hha*I, *Hinf*I, *Rsa*I and *Taq*I. The accumulation of *Taq*I repeat fragments is indicated to the right. **(B)** Agarose gel electrophoresis of the partially *Taq*I digested *Eco*RI-*Hind*III fragment isolated from cos 7 and one end-labelled at the *Hind*III site is shown.

unstable in a phage λ replacement vector or (and) the region is devoid of the restriction site *Sau*3A used for construction of genomic libraries.

To avoid a limitation of cloning probability due to an extremely reduced number of *Sau*3A restriction sites in this area the approaches considered were to clone larger fragments of partially *Sau*3A-digested DNA in cosmid vectors and to clone sheared DNA fragments *via* linker ligation in a phage lambda insertion vector. Both approaches proved to be successful. Cos 7, one out of four positive cosmid clones from a library of 5 x 10^4 colonies, which were isolated with a hybridization probe from phage λ 590, bridged the gap between λ 590 and λ 13 completely. In addition a library of cloned sheared DNA size-fractionated to an average length of 5 kb allowed the isolation of several clones with hybridization probes from the very ends of λ 590 and λ 13. Two examples, number S5 and S10, are included in Figure 2.

Identification of the DNA transcribed at 93D after heat induction

In order to demonstrate the origin of cos 7 from the heat-shock locus defined to band 93D6-7, *in situ* hybridization of nick translated cosmid DNA to polytene chromosomes of salivary gland cells was performed. Silver grains appeared exclusively at the cytogenetic map position 93D6-7, confirming the expected origin of the cloned DNA (Figure 3A). The transcription from puff 93D during a heat shock was shown by hybridization of nick-translated cos 7 DNA to heat-shocked salivary gland squashed chromosome preparations performed under conditions that favour hybridization to chromosomal RNA rather than DNA (Livak *et al.*, 1978). Only puff 93D was labelled heavily (Figure 3B). For the localization of the transcribed region within cos 7, the bridged area between phage λ 590 and λ 13 was subcloned as *Eco*RI-*Hind*III fragment (Figure 4A) and mapped with different restriction enzymes. Of eight enzymes used which recognize four or five nucleotides as target site only *Taq*I cuts within a stretch of 10–12 kb proximal to the DNA cloned into phage λ 13. Partial digests of the *Eco*RI-*Hind*III fragment (Smith and Birnstiel, 1976), labelled at the *Hind*III site that maps to the end overlapping with λ 13, with *Taq*I revealed a restriction pattern of repeated, quite regularly spaced restriction sites for *Taq*I (Figure 4B). The *Taq*I repeat structure again has been subcloned as *Hpa*II fragment into the pUC 8 vector (p*Hpa*II, Figure 4A). Digestion with *Sau*3A, *Ava*II, *Dde*I, *Hae*III, *Hha*I, *Hinf*I, *Rsa*I and *Taq*I as examples (Figures 4, lane B–I) demonstrates that all enzymes mentioned leave a fragment of ~10–12 kb uncut except *Taq*I, which accumulates a heavy band of ~280 nucleotides in length. *Taq*I restriction sites proximal to the repeat are irregularly spaced again (data not shown).

Lambda clone S5 derived from a library of sheared size fractionated *Drosophila* Oregon R DNA contained ~5.5 kb of cloned DNA. It was isolated by hybridization with the 1.5 kb *Xho*I fragment of phage λ 590 and extends from the *Xho*I fragment to ~2 kb inside the *Taq*I repeat. Restriction with *Ava*I, *Ava*II and *Eco*RI cuts the cloned S5A DNA, which has been subcloned into pUC 8 (p5A), from the vector DNA and the *Drosophila* derived part into two fragments of 2.0 and 2.5 kb and several smaller ones. Thereby it allows us to test whether the 93D heat-shock transcript derives only from the *Taq*I repeat (indicated as a bar in Figure 5) or includes the unique part of the DNA also. Hybridization of ^{32}P-labelled nuclear RNA from heat-shocked third instar larvae to Southern blots of p5A DNA restricted in this way and to DNA clones comprising ~75 kb of *Drosophila* DNA from the walk shown in Figure 2 (from λ 557 to λ 12) was performed. As shown in Figure 5, almost exclusive labelling of the 20-kb cos 7 *Hind*III fragment, which includes the area between phages λ 590 abd λ 13, and of the 5.5-kb *Eco*RI fragment from clone p5A, indicated that the transcript indeed derived from the region which was missing in the λ libraries used (see Figure 2). Strong hybridization to the 2.5 kb *Ava*I-*Ava*II fragment from p5A (fragment *b*) confines most of the transcribed region further to the *Taq*I repeat. The very weak hybridization to fragment *a* indicates that the heat shock RNA may extend into the neighbouring unique part of the sequence.

The TaqI repeat at 93D6-7 is unique in the chromosome

In situ hybridization of cos 7 DNA to polytene chromosomes from salivary glands of third instar larvae always resulted in a single site accumulation of silver grains only (Figure 3A). Since it is feasible that repetitive DNA could be underreplicated in polytene nuclei, recloned *Taq*I repeat DNA was hybridised to Southern blots of *Eco*RI digested genomic Org R DNA isolated from 0–12 h embryos and 2-day-old flies respectively. In both cases only one band of the expected size appeared (data not shown) confirming the uniqueness of the *Taq*I repeat sequences.

The only externally-repetitive sequence found within the clones spanning 85 kb of *Drosophila* DNA from region 93D

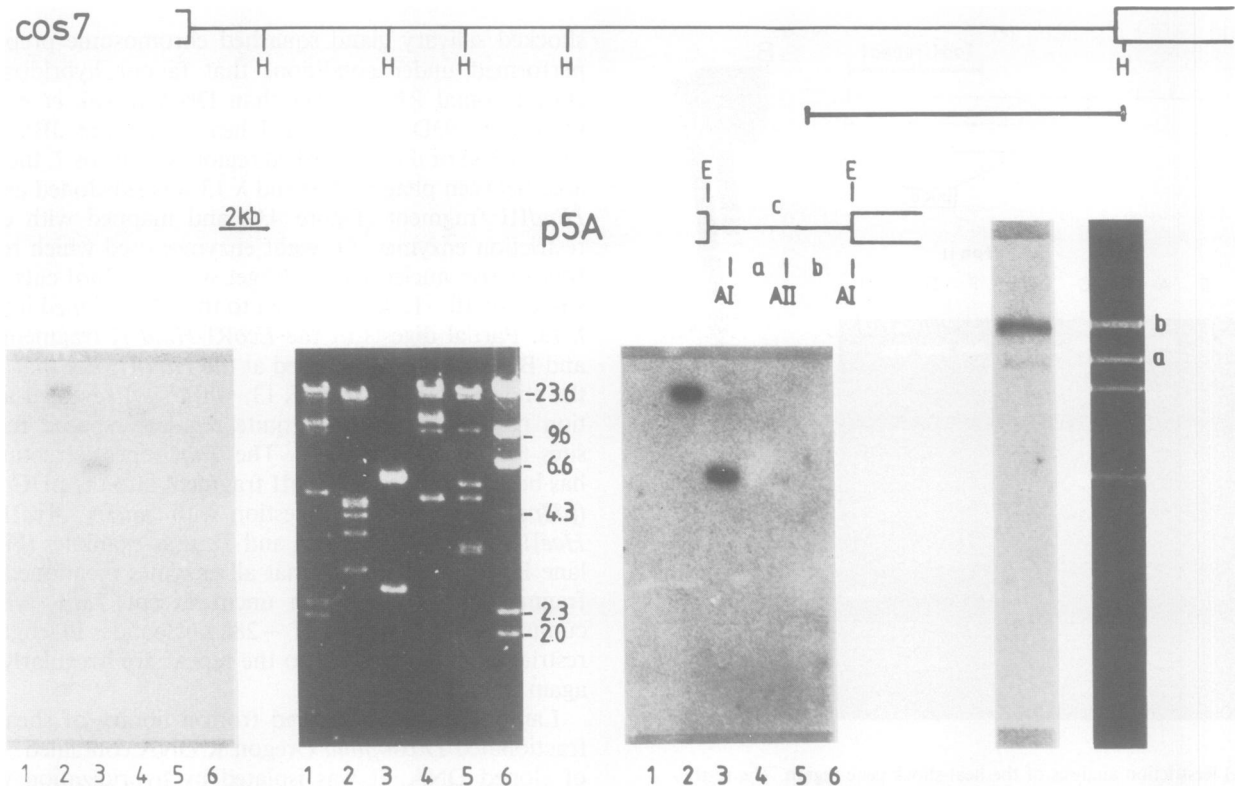

Fig. 5. Identification of the DNA transcribed after heat shock. The *Hind*III restriction sites of cos 7 DNA are shown; the bar below the map indicates the *Taq*I repeat region. P5A contains the lambda S5 DNA insert (fragment c) recloned as *Eco*RI fragment. Only the restriction sites for *Ava*I and *Ava*II, which give rise to the 2.0 kb fragment *a* and 2.5 kb fragment *b* are shown. Below the ethidium bromide staining pattern of an agarose gel containing DNA from the walk in 93D and a size marker is shown. Next to the staining pattern identical blots from this gel hybridized with nuclear RNA isolated from 24°C (left) and 36°C (right) treated third instar larvae are shown. **Lanes 1–6** contain the following DNA clones: λ 557, cos 7, p5A, λ 13, λ 12 and wt λ DNA. Cos 7 and the size marker wt lambda DNA are *Hind*III digested, all the other DNAs are *Eco*RI digested. To the right the ethidium bromide staining pattern of an agarose gel electrophoresis of *Eco*RI, *Ava*I and *Ava*II digested p5A DNA is shown together with its nitrocellulose blot hybridized with nuclear RNA from heat-shocked third instar larvae.

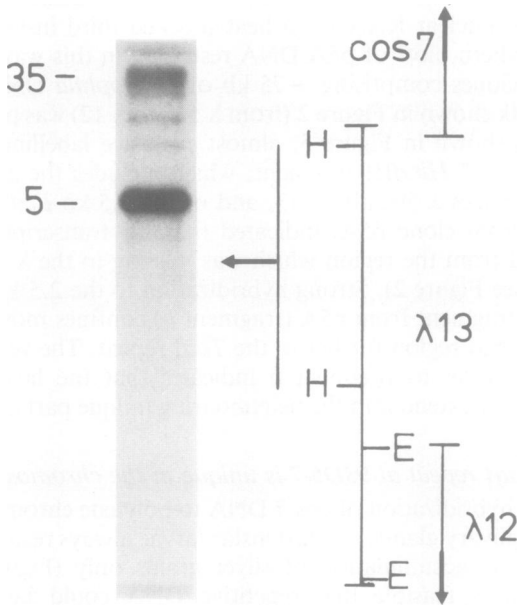

Fig. 6. Evidence for a repeated sequence. Cloned phage λ 13 DNA is shown with the overlapping DNA from the neighbouring clone λ 12 and cosmid 7. Restriction sites for *Eco*RI and *Hind*III are indicated. A 8.5 kb *Hind*III fragment used as probe for hybridization to a Southern blot of *Eco*RI digested Org R DNA is represented by the vertical line. A size scale in kb is given to the left.

is contained in the 8.5 kb *Hind*III fragment of phage λ 13. Hybridization to Southern blots of *Eco*RI digested genomic Oregon R DNA isolated from 2-day-old flies labelled a band of 5.5 kb in size and some additional minor bands including the expected *Eco*RI band of 35 kb, from where the *Hind*III fragment was derived (Figure 6). The major band seems to reflect a copy number of ~50. *In situ* hybridization with phage λ 13 revealed some grains above the chromocentre besides the unique euchromatic label at 93D6-7 (data not shown).

Discussion

We have cloned genomic DNA from heat shock locus 93D6-7 of *D. melanogaster*. *In situ* hybridization experiments map the DNA to the expected chromosomal locus. Even a longer exposure showed no cross-hybridization to other loci except for a defined region in phage λ 13, which seems to be repeated in the chromocentre. The region of transcription after heat shock is limited to a *Taq*I repeat structure and neighbouring proximal sequences (represented by fragment *a* and *b* of clone p5A in Figure 5) which are exclusively labelled after hybridization to nuclear RNA from heat-shocked third instar larvae.

Considering the genetic data available for heat-shock locus 93D, it was not surprising to clone a piece of DNA of such an unusual sequence arrangement. Since the 93D heat-shock gene area defined by the overlapping deficiencies of mutants

eGP4 and GC14 appeared to be devoid of point mutations (Mohler and Pardue, 1984), it was tempting to speculate that the 93D transcript would probably not contain a coding sequence. The overall structure of the cloned gene region is clearly compatible with the genetic analysis. The restriction map of repeated *Taq*I sites together with sequence data on several *Taq*I repeats (Hovemann, unpublished) indicate a mostly repetitive RNA transcript without coding function.

The corresponding heat-shock locus from *D. hydei* (Peters *et al.*, 1982, 1984) also contains an internally-repetitive structure and the RNA transcribed after heat shock derives from the repetitive and flanking unique part of the DNA. Although the repeat structure cloned from *D. hydei* showed no apparent cross-hybridization to the *Taq*I repeat of heat-shock locus 93D the similarity in the overall structure of small repeat units clustered over several kb in length is astonishing.

A similar case of rapid sequence divergence during evolution has been demonstrated for the organization of the non-transcribed spacers of the rRNA genes from *D. hydei* and *D. melanogaster* (Renkawitz-Pohl *et al.*, 1980). Both spacers contain a cluster of repeated sequence elements at a similar position within the spacer of both species. No sequence homology has been found between the non-transcribed spacers of *D. hydei* and *D. melanogaster* but among sibling species of the *D. melanogaster* subgroup the non-transcribed spacer remains conserved (Tartof, 1979).

A comparative situation may occur for the heat-shock loci 2-48B and 93D. It will have to be analyzed whether *Drosophila* species more closely related to *D. melanogaster* reflect a higher degree of homology between the corresponding heat-shock loci.

It has been shown previously (Dangli *et al.*, 1983) that heat-shock puff 93D from *D. melanogaster* accumulates giant particles of possible storage function as do the corresponding loci 48B and 20CD of *D. hydei* and *D. virilis* respectively. Indirect immunofluorescence studies and immunoelectron microscopy revealed a special class of RNP particles which is possibly involved in the storage of primary transcription products inside the nucleus after heat induction.

In the light of these data an RNA-containing short tandem repeats, which should have the property of binding, directly or *via* additional factors (RNA or proteins), a selected class of RNP particles, could fullfill the requirements posed by all the known phenomena associated with the action of this locus. A detailed analysis of the transcription unit in progress will help to elucidate the function of heat-shock locus 93D in more detail. In addition it may provide the tools for an *in vitro* test system including *in vitro* transcribed 93D heat-shock RNA and nuclear extracts as well as an *in vivo* test system of 93D function after P-factor-mediated transformation into the germline.

Materials and methods

General methods

Restriction endonuclease digestions, gel electrophoresis of DNA, end-labelling of DNA fragments and Southern transfer experiments were performed as described by Maniatis *et al.* (1982). For labelling of RNA we followed the protocol of Scherer *et al.* (1981), for the construction of a cosmid library of partially *Sau*3A-digested Canton S DNA in cosmid vector EMBL cos 4 we followed the protocol given by Pirrotta *et al.* (1983).

Library screening and walking

A library of partially *Sau*3A-digested genomic *D. melanogaster* Oregon R DNA cloned into the *Bam*HI site of the EMBL 4 phage lambda vector (Frischauf *et al.*, 1983) by Pirrotta *et al.* (1983) has been used for the general screening and walking procedure. $1-2 \times 10^4$ phages were grown on a 12 x 12 cm agar plate; 5–10 plates were used for one screening procedure. Phage DNA was transferred to nitrocellulose (Schleicher and Schuell, BA 85) according to Benton and Davis (1977). Filters were used without prehybridization in a hybridization solution described by Maniatis *et al.* (1978) with a nick-translated DNA probe of a specific activity of 1×10^8 d.p.m. The filters were washed twice in a 3 x SET, 0.1% SDS, 0.1% sodium pyrophosphate for 5 min at room temperature and twice in 1 x SET, 0.1% SDS, 0.1% sodium pyrophosphate for 30 min at 65°C. Exposure to Kodak XAR 5 film was performed at −70°C for ~4 h using an intensifying screen.

Preparation of genomic DNA

Flies were ground in a pre-cooled mortar to a fine powder which was further homogenized with a loose fitting Dounce homogenator. Nuclei were prepared according to standard procedures. Purified nuclei were taken up in 0.15 M NaCl/0.1 M EDTA pH 8 and sarcosyl was added to 2%. Lysis was induced by incubation at 65°C for 10 min. After addition of 3 volumes 0.15 M NaCl/0.1 M EDTA and proteinase K to a concentration of 200 μg/ml incubation at 65°C was continued for 1 h. After extraction with chloroform/isoamylalcohol (24:1), CsCl was added to the supernatant until a refration index of 1.398 was reached. Centrifugation for 20 h in a Ti 75 rotor at 40 000 r.p.m. gave a clean fraction of high mol. wt. DNA, which was dialyzed extensively against 10 mM Tris/Cl, 1 mM EDTA pH 7.5.

In situ hybridization

Chromosomes were dissected and prepared for hybridization as described by Saluz *et al.* (1983). Acetylation of the squashed chromosome preparations was performed to reduce background labelling (Hayashi *et al.*, 1978). [^{3}H]DNA with specific activity of $1-2 \times 10^7$ d.p.m./μg was prepared by nick translation according to Rigby *et al.* (1977) using all four [^{3}H]deoxynucleosidetriphosphates. 3×10^5 d.p.m. were applied per slide in a hybridization mixture containing 0.01 M Pipes buffer pH 6.8, 3 x SSC, 1 x Denhardts solution (Denhardt, 1966), 50% formamide and 2 μg sonicated carrier DNA. The hybridization was performed for 16 h at 38°C. For hybridization to chromosomal RNA *in situ* the RNase A and alkali treatments were omitted and the hybridization conditions changed to 0.01 M Pipes buffer pH 6.8, 0.4 M NaCl and 50% formamide (Livak *et al.*, 1978).

Preparation of a phage lambda library of sheared size fractionated D. melanogaster Oregon R DNA

High mol. wt. genomic DNA isolated from *D. melanogaster* Oregon R embryos was sheared with a MSE 100 sonifier for 5 s at lowest power in 300 mM LiCl. The size distribution of the DNA was tested by agarose gel electrophoresis. Usually one third of the DNA still runs as high mol. wt. DNA. The DNA was precipitated and taken up in 50–100 μl volume and size fractionated by NaCl gradient centrifugation. The linear gradient was formed with 5 and 25% NaCl solutions in 100 mM EDTA, pH 7.0 and run in a SW 40 rotor to an $\omega^2 t$ value of 5×10^{11}. DNA fragments of 4–6 kb in length were isolated from the gradient and the frayed ends were filled in with T4 polymerase. *Eco*RI restriction sites were blocked by methylation and 0.5 μg of this product was ligated with 100 pmol of *Eco*RI linker octanucleotides in a final volume of 10 μl. After extensive digestion with *Eco*RI the DNA was separated from the linker fragments on a small Sephadex G50 column and ligated to *Eco*RI cut and phosphatase-treated lambda NM 1149 vector for 16 h at 15°C. To eliminate the non-recombinant turbide plaques the library was amplified by plating on *Escherichia coli* POP101 lyc7 (Lathe and Lecocq, 1977).

Preparation of nuclear RNA

Climbing third instar larvae were heat shocked for 45 min at 36°C and immediately frozen in liquid nitrogen, control larvae were kept at 20–24°C and also quickly frozen. The frozen larvae were homogenized in lysis buffer (30 mM NaCl, 10 mM $CaCl_2$, 100 mM Tris/Cl, pH 8.5, 1% Nonidet P-40), filtered and centrifuged at 700 *g* for 10 min. The nuclear pellet was taken up in RSB (10 mM NaCl, 1.5 mM $MgCl_2$ and 10 mM Tris/Cl pH 7.2) and purified by centrifugation through 8% sucrose in RSB. The nuclei were taken up in lysis buffer (50 mM Tris/Cl pH 7.5, 200 mM NaCl, 10 mM EDTA and 1% sarcosyl) and digested with proteinase K at a final concentration of 500 μg/ml for 3 h at 36°C for heat-shocked larvae and at 20°C for the control animals. CsCl was added to a final concentration of 1 g/ml and the solution was layered over 1.2 ml of 5.7 M CsCl in 100 mM EDTA. After centrifugation at 35 000 r.p.m. for 20 h (20°C) in a SW 60 rotor only the RNA is pelleted. The RNA was taken up in H_2O bidest and precipitated.

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