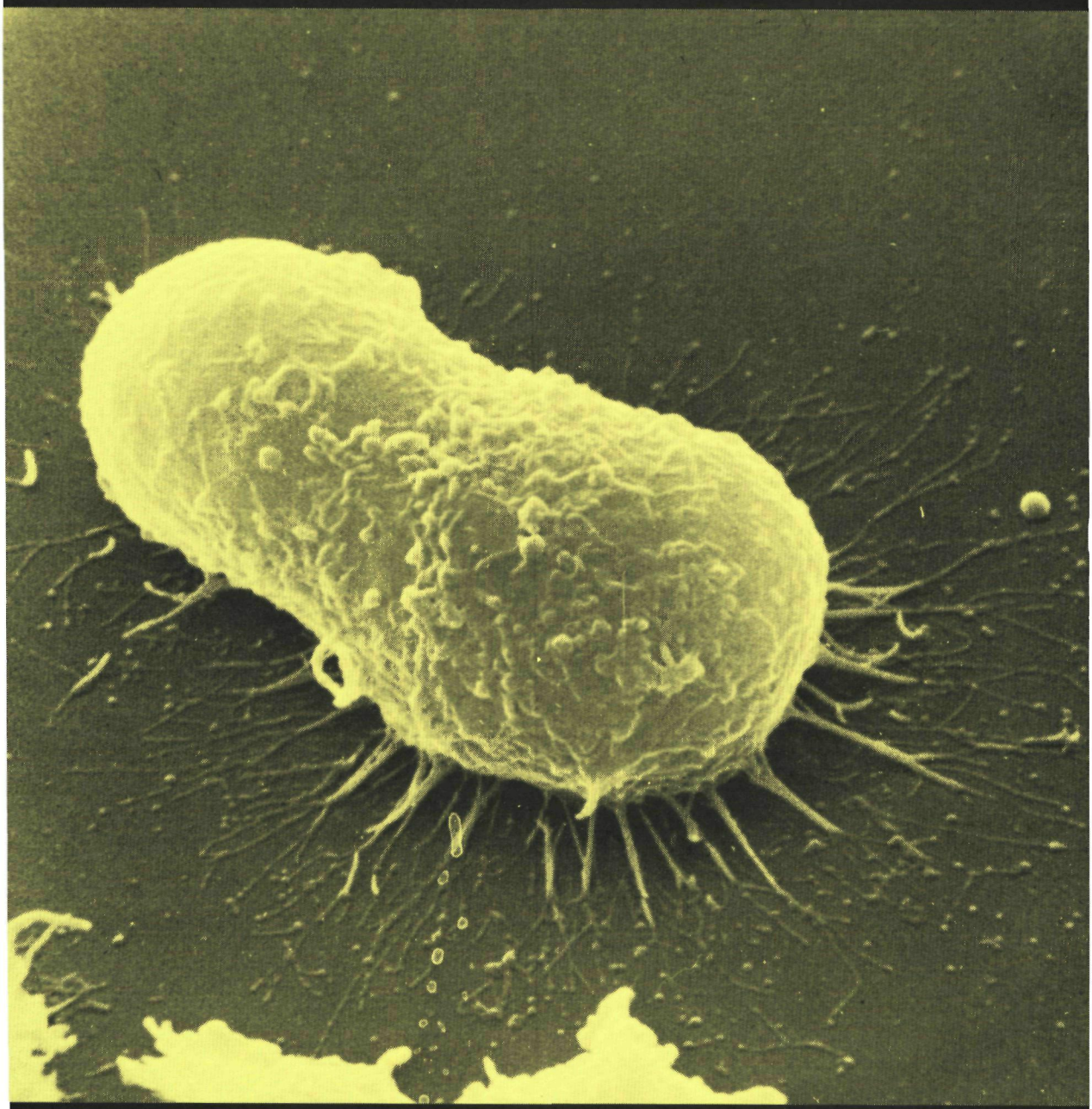
EXPERIMENTALLY INDUCED GENE ACTIVITY IN DROSOPHILA HYDEI

Characterization of RNA transcripts



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Characterization of RNA transcripts

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TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN
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Ook aan de verwezenlijking van dit proefschrift hebben uiteraard velen hun bijdrage geleverd. Zonder direct hun namen te noemen wil ik allen op deze plaats bedanken voor de vaak inspirerende medewerking, die voor mij van essentiële betekenis is geweest om dit werk te kunnen voltooien. Ook voor de afwerking van het proefschrift, die na mijn vertrek uit Holland heeft moeten plaatsvinden, wil ik alle betrokkenen bedanken. Tenslotte zou ik speciaal mijn vrouw en José van Daelen-Lammers willen vermelden die altijd met veel toewijding het type-werk hebben verzorgd.

aan Marian

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All chapters describing the experimental work have already been published

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CHAPTER I

INTRODUCTION

General

One of the most intriguing phenomena in biology is the cellular process that regulates gene expression. Most of the basic principles of gene regulation in prokaryotes are well understood these days. Some of these mechanisms also operate in eukaryotes. However, the situation in higher organisms is far more complicated due to the many developmental processes which lead to highly differentiated tissues. Furthermore, the study of gene expression in eukaryotes meets with practical difficulties: even primitive eukaryotes contain many more genes than E.coli, the prokaryote which has been the most thoroughly studied. Therefore, most data pertaining to the structure and expression of genes in eukaryotes have been obtained using cell types in which just a few genes are expressed at high levels. Examples are reticulocytes which mainly produce globin chains or lens cortex cells which synthesize large amounts of structural proteins called crystallines. Recently, in these systems many fascinating aspects of gene structure and transcription and of processing and translation of the RNA products have been elucidated. However, only little progress has been reported in studies of the regulatory mechanisms which control the induction and repression of genes in terminally differentiated cell types. Furthermore, the induction of the genes typical for the differentiated state of the tissue requires a predifferentiation of that tissue. Although the gene induction itself may be studied (for example: the secondary stimulation by estrogen in the oviduct) this predifferentiation step (for example, during the primary response to estrogen) is much less amenable to experimental study. An alternative approach to this problem is the study of the heat shock genes of Drosophila. This system offers the possibility to directly induce gene activity by relative simple experimental stimuli. In recent years, many pa-

pers have been published all concerning the complex response of Drosophila tissues to heat shock or other external inducers. I hope that the work presented in this thesis will contribute significantly to the present knowledge of the mechanisms of this response.

Heat shock in Drosophila

The history of the heat shock system starts with the observations of Ritossa (1962, 1964a), who discovered that gene activity in larval salivary glands from Drosophila can be experimentally induced as indicated by the appearance of a unique set of puffs on the polytene chromosomes. The activation of the same loci can be achieved by a wide variety of treatments, for instance by increasing the environmental temperature about 10°C, which in fact explains the name of this system, but also by agents which interfere with cellular metabolism. In D. melanogaster, the species most data refer to, there appear nine puffs at specific sites on the chromosomes after a heat shock (Ritossa, 1962; Ashburner, 1970; Ellgaard, 1972). Other Drosophila species show a similar response, for example six puffs are induced in D. hydei (Berendes et al., 1965; Leenders et al., 1973). Induction of the puffs starts within 1 min after increasing the temperature and requires RNA but not protein synthesis. Remarkable is the fact that the activity of the remainder of the chromosome, including other puffs present at the time the heat shock began, regresses simultanously (Bonner and Pardue, 1976).
Furthermore, Tissières et al. (1974) discovered another very interesting aspect of this system: a drastic change occurs in the protein synthetic pattern after a heat shock, resulting in the preferential synthesis of a specific set of polypeptides. It was found that all tissues, including the permanent cell lines, show this heat shock response at the level of RNA and protein synthesis. Thus, a heat shock induces the transcription of a specific set of RNAs, such as occurs in the puffs of the polytene chromosomes, and the major part of this RNA functions as messenger RNA and is preferentially translated into a set of polypeptides called the heat shock proteins.
The effect of heat shock is reversible unless the treatment is con-

tinued for a prolonged period of time. In their natural environment Drosophila larvae may frequently meet situations such as brief heat shock due to exposure to the sun. Therefore it is suggested that the response is homeostatic, tending to alleviate the stress situation caused by the initial stimulus. More of less analogous responses to heat shock or other inducing treatments have been described in chick fibroblasts (Kelley and Schlesinger, 1978), plants (Sachs and Freeling, 1978) and protozoa (Guttman et al., 1980), indicating that this complex response may be a common event which enables an organism to survive temporary defects in the cellular metabolism.

The induction of the heat shock genes

Besides the heat shock itself, there exist a wide variety of agents which induce a more or less comparable response in Drosophila (for review see Ashburner and Bonner, 1979). Amoung those one will find uncouplers of oxidative phosphorylation, inhibitors of electron transport, hydrogen acceptors and inhibitors of various enzymes and other cellular functions. Whether or not all these agents act via a common pathway cannot yet be decided.

Leenders et al. (1974) have suggested that the mitochondrial electron transport and oxidative phosphorylation could be the primary targets of many of the inducing agents on the basis of the following observations. The kinetic properties of several mitochondrial enzymes involved in these processes change during heat shock or recovery from anoxia. The change in specific activity of some enzymes procedes the induction of the heat shock loci and has shown to be independent of RNA synthesis which suggests that these processes are involved in the induction of the heat shock response.

For example, the increase of the app. K_m of the mitochondrial α-glycerophosphate oxidase may be related to the induction of one of the temperature sensitive loci. This change, which can be obtained with several inhibitors, such as arsenite, rotenone, antimycin and dinitrophenol, was suggested to be due to a modification of the enzyme: a protein component would be released from the complex and transmitted directly or indirectly to the nucleus and induce one of the

sensitive loci. However, the initial increase of the app. K_m of α-glycerophosphate oxidase may be a secondary effect which results from an allosteric deactivation of this enzyme due to the simultanously rapid decreasing concentration of the substrate α-glycerophosphate. This explanation is supported by the observation (Vossen, unpublished results) that addition of α-glycerophosphate prior to the puff inducing treatment reduced the change in the app. K_m of the mitochondrial α-glycerophosphate oxidase. A secondary increase in the app. K_m of this enzyme, which occurs after prolonged temperature treatment, may still be related to a modification of the α-glycerophosphate oxidase since it can be prevented with eserine sulphate, an inhibitor of carboxyl esterase.
The idea that the heat shock response would be triggered in some part of the cytoplasm is supported by the experiments of Sin (1975) and Compton and McCarthy (1978). For instance, Sin prepared a heat-labile, nondialyzable fraction from temperature treated isolated mitochondria which was capable of inducing two heat shock loci when injected in the cytoplasm of salivary gland cells.

RNA and protein synthesis during heat shock

A heat shock treatment causes three distinct responses on the level of RNA synthesis: the induction of a class of heat shock RNAs, the suppression of the synthesis of most other mRNAs and the disruption of the normal processing of the primary ribosomal RNA transcripts. (Ellgaard and Clever, 1971; Lengyel and Pardue, 1975; Rubin and Hogness, 1975; Jacq et al. 1977). Only the first effect will be discussed in detail since it has been the major point of interest in this study. One of the ways to show the transcriptional response is by incubating salivary glands with ^{3}H-uridine and preparing autoradiographs of the polytene chromosomes (Ritossa, 1964b; Berendes, 1968; Tissières, et al., 1974). These data showed that de novo synthesis of RNA takes place at the heat shock sites while preexisting developmentally regulated puffs cease to incorporate the precursor. Also the distribution of RNA polymerase and other chromosomal proteins is changed after a heat shock which again reflects a shift in

the chromosomal transcription pattern (Plagens et al., 1976; Silver and Elgin, 1977; Jamrich et al., 1977; Elgin et al., 1978). Furthermore, the accumulation of specific RNAs at the heat shock puffs can be demonstrated by hybridizing complementary DNA sequences to the puff RNA (Livak et al., 1978; chapter VI). The heat shock RNAs as they appear in the cell, have been analyzed by in situ hybridization to polytene chromosomes and by gelelectrophoresis (McKenzie et al., 1975; Spradling et al., 1975; Henikoff and Meselson, 1977; chapter III and VI). The polyadenylated fraction of the heat shock RNAs sediments as two major classes of 20S and 13S on sucrose gradients.

Further fractionation of these RNAs by electrophoresis under denaturing conditions (Spradling et al., 1977) reveals many different RNA species which can be identified by in situ hybridization.

The translational effect of a heat shock can be seen from the protein synthetic pattern as analyzed by SDS electrophoresis. The synthesis of a small number of heat shock proteins is induced while the synthesis of most other ones is repressed, although the latter effect varies among different Drosophila tissues (Tissières et al., 1974; Lewis et al., 1975; McKenzie et al., 1975; Koninkx, 1976; chapter V). The heat shock proteins are encoded by messengers synthesized only by heat shocked cultured cells, as has been demonstrated by in vitro translation (McKenzie and Meselson, 1977; Mirault et al., 1978; Moran et al., 1978; chapter V).

Furthermore, one should emphasize the extreme rapidity of the response, since one of these newly synthesized proteins can be detected within 10 min after heat shock (Mirault et al., 1978). In addition to the time required for synthesis of the messenger, it would take approximately 3-4 min to synthesize the polypeptide. Therefore, the induction of the gene, the processing of the primary transcript and the transport to the polysomes must be very rapid.

Function of the heat shock proteins

The functional significance of the heat shock proteins is probably the most ignored aspect of the system. Data obtained with inhibitors

of RNA or protein synthesis (Leenders and Beckers, 1972; Mckenzie, as quoted in Ashburner and Bonner, 1979), indicate that the heat shock polypeptides in some way regulate the activity of the heat shock genes. The expected regression of the heat shock puffs some time after induction did not occur when glands were incubated with cycloheximide. Also, cells which have been treated with actinomycin-D during heat shock do not recover their normal protein synthetic pattern when returned to 25°C. These data suggest an autoregulatory effect: a heat shock induces the synthesis of a set of mRNAs coding for proteins which enable the cell to overcome the metabolic distortion caused by the treatment. A new equilibrium is reached and the activity of the induced genes, which are no longer needed, will decrease.
Based on this assumption, studies have been made of the effects of heat shock and anoxia on the levels and kinetic properties of several enzymes involved in respiratory processes. Changes have been reported in mitochondrial NADH dehydrogenase, tyrosine aminotransferase and isocitrate dehydrogenase (Leenders et al., 1974; Koninkx, 1975 et al., 1975; Sin and Leenders, 1975). However, the observed decrease in the app. K_m of these enzymes may not simply be due to de novo synthesis of the enzymes. For instance the increase in the activity of the cytoplasmic α-glycerophosphate dehydrogenase can be prevented by eserine sulphate, an agent which inhibits deacetylation. Furthermore, it has been found that the same change in app. K_m of α-glycerophosphate dehydrogenase can be obtained in the control extracts by mixing them with extracts of heat shocked material (Vosson, unpublished). These data indicate that modification may be the essential mechanism by which the cell regulated the response on the respiratory level after a metabolic disturbance such as anaerobiosis. Whether the heat shock proteins, or at least some of them, act as modifying enzymes or whether they induce secondary processes which finally lead to the modification of the aforementioned enzymes cannot yet be decided. To resolve the function of the heat shock proteins, some have suggested a genetic approach (Ish-Horowicz, 1980). If indeed the normal response to a heat shock enables the animal to withstand the metabolic stress then it may be possible to use this feature when screening for mutants of the temperature sensitive loci. However, conclusive data which would support this suggestion, have

not yet been reported.

Heat shock in D. hydei

So far, this introduction has presented a more general survey of the heat shock system in Drosophila. However, the results in this thesis have not been obtained with D. melanogaster, the species to which almost all data in the literature refer. A number of reasons justify the use of D. hydei instead. Although the heat shock response in this species appears to be homologous to that in D. melanogaster, there is one major difference which concerns a locus close to the telomere of the second chromosome indicated as 2-48B on the cytological map of the polytene chromosome of D. hydei. Several observations have been made which demonstrate that this particular locus behaves differently from any heat shock locus of D. melanogaster. First, locus 2-48B produces a complex RNP particle (Derksen et al., 1973) after heat shock. Such particles cannot be found in any of the temperature sensitive loci in D. melanogaster. In D. hydei, these particles also appear after incubation with vitamin B6 while in D. melanogaster non of the heat shock puffs can be induced with this treatment.

The properties of locus 2-48B of D. hydei can be summarized as follows: analyses of heat shock RNA present in the nucleus and cytoplasm showed that 80% of the 2-48B sequences remains in the nucleus while the rest, containing non-homologous RNA sequences, can be found associated with the polysomes (chapter III, VI and VII).
Previous analyses by micromanipulation of heat shock or vitamin B_6 induced salivary glands showed the presence of a 40S RNA transcript in the puff at locus 2-48B (Bisseling et al., 1976). These exceptional features caused locus 2-48B in D. hydei to become a major point of interest of this thesis. By the time this work was initiated permanent tissue culture cell lines of D. melanogaster had already been isolated (Kakpakov et al., 1969; Echalier and Ohanessian, 1970). These cell lines proved to be very useful to study RNA transcription during heat shock. Unfortunately, no such cell lines were available for D. hydei and therefore they were isolated at the laboratory in

Nijmegen. Their morphology and karyotype are described in chapter II and these cells were used for the experiments in the following chapters as a convenient tissue in which to study the heat shock response. The RNA transcripts, homologous to locus 2-48B, were analyzed by hybridization of pulse labelled nuclear and polysomal RNA isolated from heat shocked cultured cells (chapter III). The following chapters (IV, V) will describe the coding properties of the heat shock RNAs as they appear associated with the polysomal fraction in the cell. The data presented in chapter IV suggest a correlation between one of the heat shock proteins, i.e. the 70,000 dalton polypeptide, and locus 2-36A in D. hydei. From a more general point of view, the protein synthetic patterns of different tissues during heat shock are subjected to a closer examination in chapter V. Two-dimensional analysis of the heat shock proteins reveals some tissue-specific differences and also the existence of a class of minor heat shock proteins some of which may be structurally related to the major ones. Furthermore, in this chapter the experiments are described which show that the heat shock proteins are encoded by de novo synthesized mRNA species. Whether the same mRNAs are indeed transcribed from the heat shock sensitive loci can only be decided from in situ hybridization with in vivo pulse labelled RNA if one assumes that the activity of these loci under normal conditions can be neglected. However, this did not prove to be the case for locus 4-81B as will be shown in chapter VI. The hybridization experiments, using cDNA prepared from heat shock RNA instead of in vivo labelled RNA, also showed that an additional locus (4-85B) is activated during a heat shock. With respect to locus 2-48B, the data indicated a complex structure for the presumptive nuclear transcript.

Whether the sequence of this transcript is essential to its function in the heat shock response, if any, may be answered if one considers the evolutionary stability of this feature. Therefore, we compared the presence of the heat shock sequences, especially the one homologous to 2-48B, between several related Drosophila species (chapter VII) and showed that these sequences are not conserved. In the summary the major conclusions of this thesis will be discussed also connection with some of the data obtained more recently.

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CHAPTER II

New Cell Line

ESTABLISHED CELL LINES OF *DROSOPHILA HYDEI*

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SUMMARY

Four cell lines have been isolated from *Drosophila hydei* embryos. Three lines have a normal XY karyotype, the fourth has an XO karyotype with an additional small heterochromatic fragment. The cells contain presumable cytoplasmic virus like particles.

Key words: Drosophila hydei; dipteran cell line; virus like particles.

During the last 10 years a number of *Drosophila melanogaster* cell lines have been isolated (1). Using the methodology established in these studies, we have isolated four cell lines from *D. hydei*. Cells obtained from mechanically fragmented 0- to 10-hr old *D. hydei* embryos were cultured in the M-3 medium described by Shield and Sang (2). After 4 weeks most of the flasks contained clumps of growing cells and these were subcultured without dilution. A period of very slow proliferation followed. No attempt was made to subculture the cells during this time, but once a month half of the medium was removed and replaced with fresh medium. In this lag phase a protozoan contamination, a *Gregarinea* species, appeared in practically all our cell cultures. To reduce their concentration, the medium was removed once a week, Millipore filtered, and returned to the flask. Once cell proliferation resumed, this contaminant disappeared.

After 6 months a continuous sheet of cells started to form in about 20% of the flasks. These cultures were subcultured, again without dilution, every 4 or 5 weeks until cell growth resumed without lag after each passage. Parent cultures were then split 1:1 or 1:3 for a further 10 passages. In our hands the plating efficiency of the cells remained very low during the first 10 passages and subculturing at high density proved essential to maintain viability of the cells.

Starting with 50 primary cultures in November 1975, we finally obtained four cell lines in the fall of 1976. These lines can be subcultured at a 1:10 dilution and have a doubling time of 1 to 2 days at 25° C. They have been designated KUN-DH-14, KUN-DH-15, KUN-DH-33, and KUN-DH-47; the number indicates the number of the original primary culture from which the line is derived. Although the cell lines were isolated and are maintained as monolayer cultures, they can be easily adapted to growth in suspension or spinner culture, where they reach a density of 2×10^5 or 5×10^6 cells/ml, respectively.

The karyotype of the cells is essentially normal (Fig. 1): three of the four lines are normal diploid XY, whereas line KUN-DH-14 has a diploid XO karyotype with an additional small heterochromatic fragment, which may be derived from the Y chromosome. About 10% of the cells in all lines

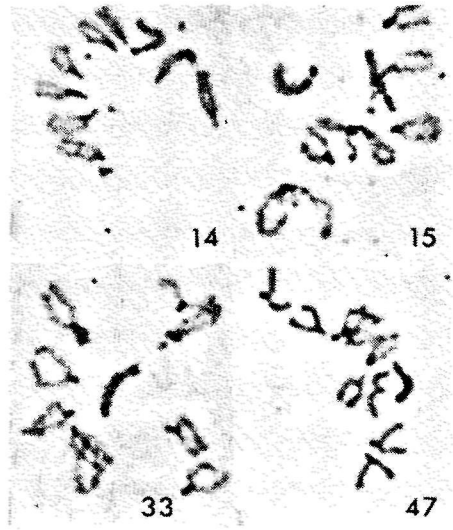

FIG. 1. Karyotypes of the four cell lines, the numbers in the figure indicate the cell line. No colchicine or vinblastine block was used since the chromosomes became difficult to recognize. Orcein stain. ×7420.

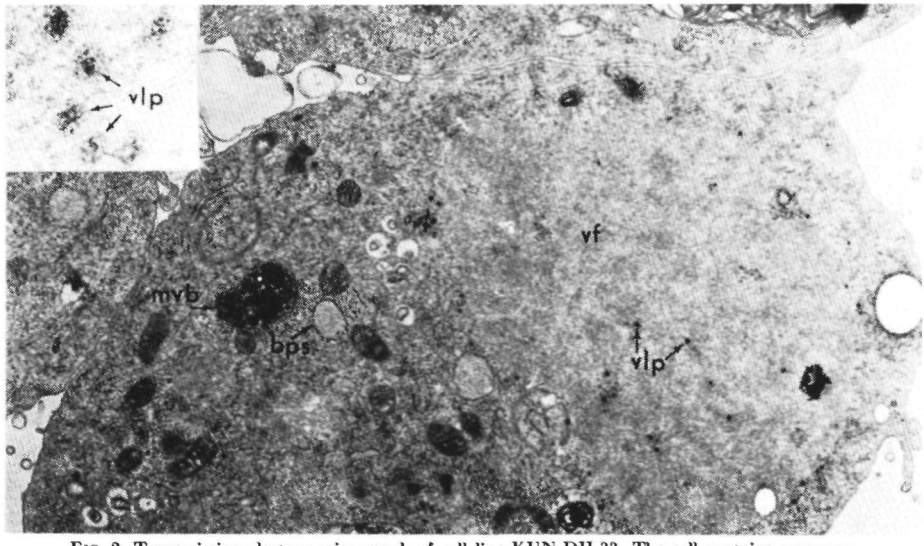

FIG. 2. Transmission electron micrograph of cell line KUN-DH-33. The cell contains numerous mitochondria, some multivesicular bodies (mvb), and bodies that may have been excluded from the perinuclear space (bps). A large virus focus (vf) surrounded by viruslike particles (vlp) occupies part of the cytoplasm. ×17,000. *Insert:* viruslike particles at higher magnification. ×82,000. Aqueous uranyl acetate and lead citrate stain.

are tetraploid. There was no change in ploidy of the cells in spinner or suspension culture.

The cells are morphologically quite similar to the small and round cells of the *D. melanogaster* K_c line isolated by Echalier and Ohanessian (3). However, in phase contrast microscopy, many cells show a large distinct oval body in the cytoplasm. In electron micrographs these structures stained less dense than the surrounding cytoplasm and contained darkly stained particles with a diameter of 600 to 800 Å at the edge of the body (Fig. 2). At higher magnification an inner core with a diameter of 300 Å can be discerned but no well defined outer edge is seen (Fig. 2, *insert*). These particles have also been seen free in the cytoplasm but have never been found in the nucleus. Budding of these presumable viruslike particles has never been observed, nor has a cytopathic effect been noted. The presence of both nuclear and cytoplasmic viruses has been reported in most of the *D. melanogaster* cell lines (1,4). The presumable virus found in *D. hydei* cells may be endogenous, comparable to, for example, sigma virus, the growth of which is supported by *D. melanogaster* embryonic cells (5). Alternatively, they may originate from the serum, as suggested for the orbivirus found in some of the *D. melanogaster* cell lines (4).

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CHAPTER III

In situ Hybridization of Nuclear and Cytoplasmic RNA to Locus 2-48BC in *Drosophila hydei*

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Abstract. The maximum grain density over the "heat-shock" locus 2-48BC of *Drosophila hydei* polytene chromosomes obtained after in situ hybridization of nuclear RNA extracted from tissue culture cells labelled during incubation at 37° C is five times higher than that obtainable by using polysomal RNA isolated from the same cells. Furthermore, the addition of a large excess of unlabelled polysomal RNA reduced the amount of in situ hybridization of nuclear RNA by only 20% showing that nuclear 2-48BC RNA contains sequences not present in polysomal 2-48BC RNA. — The polysomal 2-48BC RNA is polyadenylated, as are the RNA sequences present in the polysomes complementary to the other two major 'heat shock' loci 2-32A and 2-36A. Polyadenylated RNA, with an apparent size of 15S, complementary to locus 2-48BC is also found in the cytoplasm of *D. hydei* salivary glands.

Introduction

A specific set of puffs ("heat-shock" puffs) can be experimentally induced in the polytene chromosomes of salivary glands of *Drosophila* by a variety of treatments such as an incubation of the cells at 37° C (heat shock) or release from anaerobiosis (Ritossa 1962, 1964; van Breugel, 1966; Ashburner, 1970; Leenders and Berendes, 1972). The presence of these puffs is correlated with a change in the protein synthetic pattern: a specific set of newly synthesized peptide bands (the "heat-shock" bands) appears (Tissieres et al., 1974; Lewis et al., 1975; Koninkx, 1976). Treatments that induce puff formation and the corresponding shift in protein synthetic pattern in salivary glands also cause a similar change in protein synthetic pattern in diploid tissues and tissue culture cells (Tissieres et al., 1974; McKenzie et al., 1975). Furthermore, *D. melanogaster* cells in tissue culture synthesize RNA during heat-shock that hybridizes back to the "heat shock" sensitive loci in situ, indicating that identical loci are activated in different tissues during incubation at 37° C (McKenzie et al., 1975; Spradling et al., 1975; Bonner and Pardue, 1976; Spradling et al., 1977).

The RNA species complementary to the "heat-shock" loci found in the cytoplasm of *D melanogaster* cells have a size of 12 to 20S, contain poly A and are found in the polysomes (McKenzie et al., 1975, Spradling et al., 1975, Spradling et al., 1977) These loci thus produce mRNA although there is as yet no direct evidence for the suggestion that these are the mRNAs coding for the heat-shock bands

In *D hydei* there are four major temperature inducible loci (2-32A, 2-36A, 2-48BC and 4-81B), which are probably analogous to the "heat shock" loci of *D melanogaster* (for review see Berendes, 1975) The RNA product of only one of these loci has been studied the puff at locus 2-48BC contains 40S RNA which is not polyadenylated (Bisseling et al., 1975) Furthermore, a small molecular weight RNA (3 4S) which hybridized in situ to locus 2-48BC could be isolated from salivary glands cultured in vitro (Berendes et al., 1973)

We have further investigated the properties of the RNA product of this locus, especially with regard to a possible mRNA function, and show here RNA complementary to locus 2-48BC can be found polyadenylated and associated with polysomes in the cytoplasm However, some of the RNA produced by this locus is not transported to the cytoplasm since nuclear 2-48BC RNA contains sequences not found in polysomal 2-48BC RNA

Materials and Methods

Isolation of Radioactive RNA from Embryonic Cells

Cells were obtained from primary cultures established from 12 h old *Drosophila hydei* embryos essentially as described by Shields and Sang (1970) except that the trypsinization was omitted Cells were plated in revised Shields and Sang (pers comm) medium in 81 cm^2 plastic tissue culture flasks Usually two flasks of a five days old primary culture were used per experiment Alternatively a *D hydei* cell line established in our laboratory (Sondermeijer and Lubsen in prep) and growing in spinner culture was used as a source of material For the isolation of labelled RNA 40 µC ^{3}H uridine (48 Ci mM) 40 µC ^{3}H adenosine (33 Ci mM) 20 µC ^{3}H cytidine (26 Ci mM) and 20 µC ^{3}H guanosine (10 Ci mM) were added per ml of medium (cells growing in spinner culture were concentrated to 5×10^7 cells ml) the cells were then shifted to 37° C and incubated at 37° C for 60 min 5 min before harvesting the cells 100 µg/ml cycloheximide was added to prevent polysome run-off Cells were rapidly chilled collected by centrifugation and lysed as described by McKenzie et al (1975) except that the lysate was homogenized in an all-glass homogenizer to reduce cytoplasmic contamination of the nuclear pellet A crude nuclear pellet was obtained by centrifugation of the homogenate for 10 min at 3 000 rpm The pellet was resuspended in 0 5 mg protease K ml in 10 mM Tris HCl (pH 7 4) 0 1 M NaCl 10 mM EDTA and 1% SDS The suspension was extracted repeatedly with two volumes of phenol-chloroform DNA was removed by spooling with a glass rod and RNA was collected by precipitation from 70% ethanol The RNA pellets were dissolved in a small amount of 2×SSC and used for in situ hybridization

Polysomes were prepared from the post nuclear supernatant as described by McKenzie et al (1975) The appropriate part of the sucrose gradient was collected and the polysomes were recovered by centrifugation overnight at 45 000 rpm in an IEC A 431 rotor Alternatively polysomes were collected directly from the post nuclear supernatant by centrifugation through 1 ml of 1 M sucrose in gradient buffer for 2 5 h at 55 000 rpm in an IEC SB 405 rotor No difference in in situ hybridization patterns between RNA prepared from polysomes isolated by these two procedures was seen

Polysomal RNA was prepared from the polysomal pellets as described above for nuclear RNA For the isolation of poly A containing RNA RNA was dissolved in 10 mM Tris HCl

(pH 7.4) 0.1 M NaCl 10 mM EDTA and 0.5% SDS and heated for 2 min at 80° C. NaCl was added to 0.5 M and the mixture was passed over 0.5 ml poly U Sepharose column. The column was washed with three column volumes of 10 mM Tris HCl (pH 7.4) 10 mM EDTA 0.5% SDS 0.1 M NaCl and then eluted with two column volumes of 90% formamide at 65° C. The eluted RNA was collected by ethanol precipitation and dissolved in a small amount of 2 × SSC.

Isolation of Radioactive RNA from Salivary Glands Incubated in vitro

Salivary glands of early third instar larvae of a laboratory stock of *Drosophila hydei* were used. RNA for hybridization in situ was obtained from 100 salivary gland pairs incubated in 300 µl of Poels medium (Poels 1972) for 20 h. 4 h before the end of this period the medium was covered with liquid paraffin or saturated for 60 min with N_2. The liquid paraffin and the medium were then removed and the glands were incubated in 50 µl of fresh medium containing 20 µCi of each of the four labelled nucleosides (spec. act. 8 ^{3}H adenosine 25 Ci/mM 5 ^{3}H cytidine 28 Ci/mM 5,6-^{3}H uridine 45 Ci/mM, 8-^{3}H guanosine 13 Ci/mM) for 3 h. At the end of the incubation period the medium was removed and the glands rinsed for 1 min in 1 ml of fresh medium. The glands were homogenized for 5 min at 4° C in 1 ml of 10 mM Tris HCl (pH 7.2) 50 mM NaCl 10 mM $MgCl_2$ 0.5% NP_{40} and 40 µl diethylpyrocarbonate containing 100 µg *E. coli* RNA. The nuclei were pelleted at 3,500 rpm for 5 min. The supernate was extracted three times with two volumes phenol chloroform (1:1). Two volumes of ethanol were added to the aqueous phase and the mixture was stored at −28° C. The precipitate was collected by centrifugation dissolved in 0.3 ml of the same buffer without detergent and diethylpyrocarbonate and incubated with 40 µg/ml DNase I for 30 min at 37° C. After a phenol chloroform extraction the RNA was precipitated at −28° C by the addition of two volumes of ethanol.

The RNA was dissolved in 50% formamide − 3 × SSC (SSC is 0.15 M NaCl 0.015 M Na citrate pH 7.2) for in situ hybridization or in CSB (0.7 M NaCl 50 mM Tris-HCl (pH 7.5) 10 mM EDTA in 25% formamide) for chromatography on Poly-U-Sepharose.

For the preparation of poly A-containing RNA RNA extracted as indicated above and dissolved in CSB was applied to a Poly-U-Sepharose column. The column was washed with three bed volumes of CSB and once with CSB without formamide. The poly A containing RNA was eluted with redistilled water (pH 7) at 50° C in a water jacketed column. The eluate was lyophilized and the RNA dissolved in 50% formamide − 3 × SSC. No poly U eluted from the column after washing with water at 50° C.

Gel Electrophoresis

The size of the poly A-containing RNA was analysed on 2.7% polyacrylamide gels (Weinberg et al. 1967). Electrophoresis was performed for 40 min at room temperature and 4 mA per gel. The gels were sliced with a Mickle gel-slicer. The slices were digested overnight at room temperature with NH_3. The NH_3 was evaporated and the radioactivity measured in 10 ml of Butyl PBD-ethoxy ethanol-toluene scintillation fluid.

In situ Hybridization

Hybridization in situ of salivary gland RNA was performed as previously described (Alonso 1973). The poly A containing RNA or total RNA dissolved in 10 µl 50% formamide 3 × SSC was placed over the best 20–30 chromosomes sets selected under a light microscope. The slide was placed in a sealed plastic Petri dish containing 10 ml of the solution used to dissolve the RNA and incubated for 18 h at 30° C.

The hybridization of diploid cell RNA was performed as described by Pardue and Gall (1975) except that 2 µl aliquots of the RNA solution were placed over the squashes and covered with a round coverslip (1 cm diameter). The coverslip was sealed with rubber cement and the slides were incubated at 65° C for 18 h. Exposure times varied from 14 to 60 d depending on the source and concentration of the RNA and the locus to be analyzed. For quantitative analyses RNA

was hybridized in situ at three or four different concentrations and, after the appropriate exposure time, grains over the various loci were counted (using about ten nuclei per slide), the grain counts were corrected to a 14 d exposure time and plotted in a double reciprocal plot against RNA concentration. The best straight line was calculated using the "least squares" methods using each grain count as a single data point.

Materials

^{3}H-uridine, ^{3}H-adenosine and ^{3}H-guanosine were obtained from the Radiochemical Centre, Amersham. ^{3}H-adenosine was from New England Nuclear. Poly U-Sepharose from Pharmacia, protease K from Merck, DNase I from Boehringer, and *E. coli* rRNA from Miles.

All other chemicals used were reagent grade.

Results

It has been reported that heat shock treatment of *D. melanogaster* cells activates qualitatively the same loci irrespective of the tissue used although the quantitative response, as determined by analysis of RNA sequences via in situ hybridization (Bonner and Pardue, 1976) or as shown by the changes in protein synthesis pattern, may differ markedly. Similarly, in *D. hydei*, embryonic cells synthesize the "heat-shock" peptides during incubation at 37° C (Berendes, 1975) and the typical nuclear RNP product of locus 2-48BC is found in such cells after incubation with vitamin B_6 (Derksen, 1975) which causes puffing of this locus

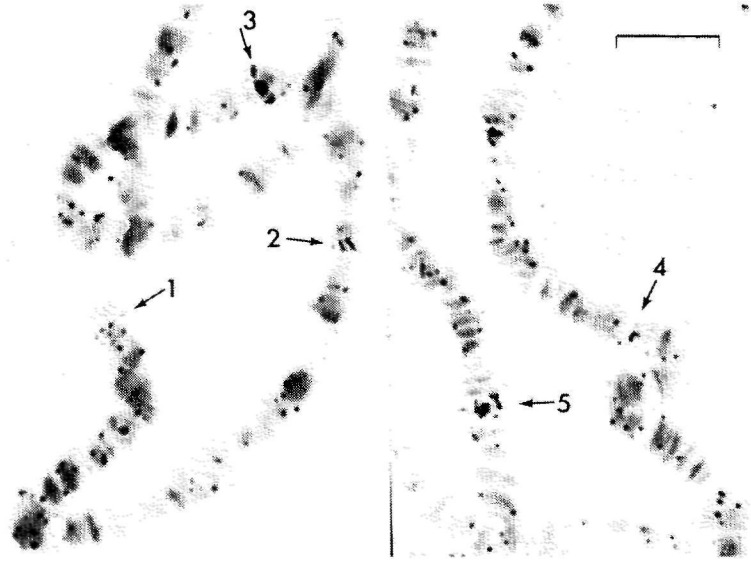

Fig. 1 [1]. In situ hybridization of polysomal RNA. Polysomal RNA was used for in situ hybridization at a concentration of 9×10^4 cpm/μl. Exposure time was 30 days. The loci are indicated as follows: 1: 2-48BC, 2: 2-36A, 3: 2-32A, 4: 4-81B and 5: 3-59A

[1] Bars in Figures 1, 2, 6 and 8 represent 10 μm

in salivary gland cells (Leenders et al., 1973). It is thus expected that the "heat-shock" loci become active during incubation of *D. hydei* embryonic cells at 37° C. This is indeed the case as shown by the pattern of in situ hybridization of total cytoplasmic RNA or polysomal RNA isolated from such cells incubated for 60′ at 37° C in the presence of ^{3}H-nucleosides: over two of the four major heat-shock loci (2-32A and 2-36A) a high grain density is seen. Locus 2-48BC also contains label but the fourth major heat-shock locus, 4-81B, only occasionally shows a grain density above that of the chromosomal background (Fig. 1). We also find strong labeling over locus 3-59A (not a "heat-shock" locus).

The same loci, i.e. 2-32A, 2-36A, 2-48BC and 3-59A, are also labelled when nuclear RNA extracted from cells labelled during "heat-shock" is used for in situ hybridization (Fig. 2a). The grain density over region 2-48BC in such preparations was always much higher than that seen when cytoplasmic or polysomal RNA (compare Fig. 2a and b) was used for in situ hybridization indicating the possibility that the nuclear RNA sequence complementary to locus 2-48BC was longer than the cytoplasmic one. Alternatively, the relative concentration or the specific activity of the nuclear 2-48BC RNA could be much higher. To discriminate between these possibilities, either nuclear or polysomal RNA was hybridized in situ at various concentrations, and hybrid formation was

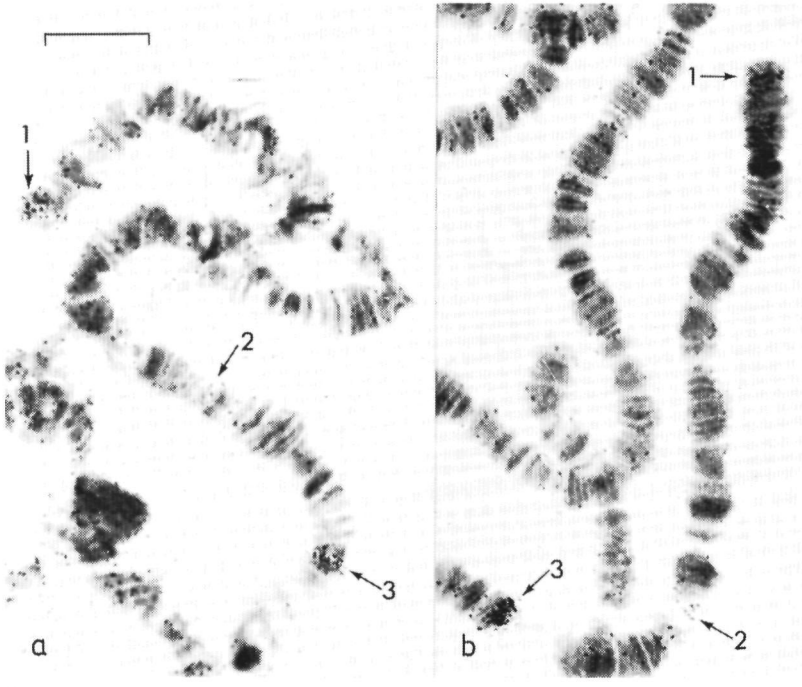

Fig. 2a and b. In situ hybridization of nuclear and polysomal RNA. Nuclear and polysomal RNA were isolated from the same batch of cells and used for in situ hybridization as described in Methods. The heat-shock sensitive loci are indicated as in Figure 1. **a** Nuclear RNA (4.4×10^5 cpm/μg) at a concentration of 5.3×10^4 cpm/μl. Exposure time was 24 days. **b** Polysomal RNA (6.3×10^4 cpm/μg) at a concentration of 3.0×10^4 cpm/μl and the exposure time was 24 d

quantitated by counting the silver grains over various loci The grain counts were then plotted in a double-reciprocal plot against the RNA concentration (Bishop, 1969) The slope of the line obtained is a measure of the relative RNA concentration while the intercept is a measure of the amount of DNA complementary to that RNA The results of such an experiment for loci 2-32A, 2-36A and 2-48BC are shown graphically in Figure 3 and the parameters of the lines are given in Table 1 These data show that the maximum grain density over locus 2-48BC that can be obtained with nuclear RNA is about five times as large as that to be expected if polysomal RNA is used The error in grain counts from autoradiograms is inherently high, especially when grain counts are low, as is the case for locus 2-48BC when polysomal RNA is hybridized in situ Nevertheless, the difference between the intercepts of the nuclear and polysomal RNA concentration curves is statistically highly significant ($p < 0.005$) In case of loci 2-32A and 2-36A, the intercept obtained using nuclear RNA is also always somewhat smaller than that obtained when polysomal RNA is used, but these differences are statistically not highly significant

A difference in intercept would also be found if nuclear RNA complementary to 2-48BC has a much higher specific activity than polysomal 2-48BC RNA. This possibility can be excluded if it can be shown that polysomal 2-48BC RNA competes only partially with nuclear 2-48BC RNA while nuclear 2-48BC should compete completely with polysomal 2-48BC RNA Accordingly, unlabelled polysomal and nuclear RNA was prepared At the same time, labelled polysomal and nuclear RNA was prepared from an identically treated, second batch of cells The relative concentrations of the various RNA species complementary to loci 2-48BC and 2-32A were determined using the labelled RNA The parame-

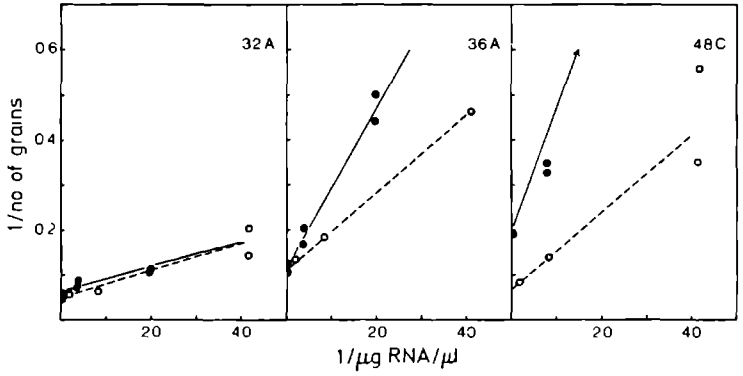

Fig. 3. Concentration dependency of the extent of hybridization of nuclear and polysomal RNA Nuclear and polysomal RNA were isolated as described in Methods from the same batch of cells and used for in situ hybridization Nuclear RNA had a specific activity of 4.4×10^3 cpm/μg and was used at concentrations between 2.7×10^3 to 1.1×10^4 cpm/μl Polysomal RNA with a specific activity of 6.3×10^4 cpm/μg was used at concentrations between 7.9×10^4 and 3.2×10^5 cpm/μl Hybridizations were quantitated as described in Methods ○---○ Nuclear RNA ● ● polysomal RNA The data shown in the figure are the average grain counts of the various slides The parameters of these lines as shown in Table 1, were calculated by the 'least squares' method using each grain count as single data point

Table 1. Concentration dependency of the extent of hybridization of nuclear and polysomal RNA. Conditions of in situ hybridization and RNA isolation as described in Methods and in the legend to Figure 3. The lines whose parameters are given in this table are shown graphically in Figure 3. n indicates the number of nuclei counted

RNA	Locus	n	Intercept (grains $^{-1} \times 10^{-1}$)	Slope (μg μl $^{-1}$)
Polysomal	32A	46	0.6 ± 0.07	$2.68 \pm 0.51 \times 10^{-3}$
	36A	40	1.1 ± 0.01	$2.79 \pm 0.95 \times 10^{-2}$
	48C	36	2.0 ± 2.0	$9.13 \pm 2.49 \times 10^{-2}$
Nuclear	32A	23	0.4 ± 0.2	$3.00 \pm 0.54 \times 10^{-3}$
	36A	19	1.0 ± 1.8	$1.82 \pm 0.58 \times 10^{-2}$
	48C	21	0.7 ± 1.0	$1.06 \pm 0.31 \times 10^{-2}$

Table 2. Concentration dependency of the extent of hybridization of nuclear and polysomal RNA. Nuclear RNA (3.6×10^{5} cpm μg) in concentrations ranging from 0.05 to 0.35 μg μl and polysomal RNA (4.1×10^{4} cpm μg) in concentrations ranging from 0.4 to 2.95 μg μl were used for in situ hybridization. The extent of hybridization was determined as described in Methods. n indicates the number of nuclei counted

RNA	Locus	n	Intercept (grains $^{-1}$)	Slope (μg μl $^{-1}$)
Polysomal	32A	28	$5.11 \pm 0.51 \times 10^{-3}$	$1.55 \pm 0.31 \times 10^{-2}$
	48C	47	0.43 ± 0.12	$4.76 \pm 7.33 \times 10^{-2}$
Nuclear	32A	53	$4.88 \pm 0.47 \times 10^{-2}$	$1.33 \pm 0.38 \times 10^{-3}$
	48C	58	$6.24 \pm 0.99 \times 10^{-3}$	$2.37 \pm 0.80 \times 10^{-3}$

ters of these lines are given in Table 2. Then in situ hybridization was performed using a fixed amount of labelled RNA and increasing amounts of unlabelled competitor RNA. The resulting grain densities over loci 2-32A and 2-48BC expressed as percent of control and plotted against the ratio of total RNA to labelled RNA are shown in Figure 4. Locus 2-32A served as a control in this experiment. It can be calculated from the slopes of the lines given in Table 2 that nuclear RNA in this experiment contained about twelve times as much 2-36A RNA on a weight basis as did polysomal RNA. A 50% reduction in grain counts over this region is thus expected with a twelve fold excess of unlabelled polysomal RNA over labelled nuclear RNA. From the data given in Figure 4 a fifteen fold excess appears to be required and the competition by polysomal RNA is thus about as efficient as expected. In contrast to the results obtained for locus 2-32A, polysomal RNA competes very poorly with nuclear 2-48BC RNA: only a 20% reduction in the grain count is seen even if unlabelled polysomal RNA is present in a fifty fold excess over labelled nuclear RNA. This lack of competition is not due to too low a concentration of 2-48BC RNA in the polysomal preparation, since the reverse experiment, namely the competition of labelled polysomal RNA with unlabelled nuclear RNA, shows that the concentration of these species is about equal: the addition of an equal amount of unlabelled nuclear RNA to labelled polysomal RNA

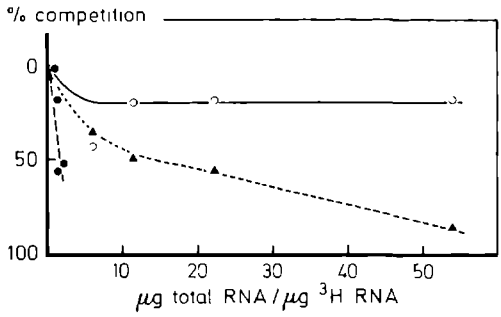

Fig. 4. Competition between nuclear and polysomal RNA in situ hybridization. Nuclear RNA (3.6×10^5 cpm/μg, 0.12 μg/μl) or polysomal RNA (4.1×10^4 cpm/μg, 0.99 μg/μl) was hybridized in situ in the presence of increasing amounts of either unlabelled polysomal or nuclear RNA as indicated in the figure. ○---○ hybridization to locus 2-48BC of nuclear ^{3}H-RNA. ▲---▲ hybridization to locus 2-32A of nuclear ^{3}H-RNA. ●---● hybridization to locus 2-48BC of polysomal ^{3}H-RNA

yields a 50% reduction in grain density (Fig. 4). These data then show that the amount of DNA complementary to nuclear 2-48BC RNA is higher than the amount of DNA complementary to polysomal 2-48BC RNA.

Polyadenylation of Polysomal 2-48BC RNA

The data presented above indicate that sequences complementary to loci 2-32A, 2-36A and 2-48BC are present in polysomal material and that their presence cannot be due to nuclear leakage since there is a marked difference between nuclear and polysomal 2-48BC RNA. To determine whether these RNA species are polyadenylated, poly-A containing RNA was prepared from polysomal RNA by poly-U-Sepharose chromatography and the relative concentration of these sequences in this material as compared to the total polysomal RNA was determined. The data obtained are shown graphically in Figure 5: the grain counts obtained using total polysomal RNA are again plotted in a double reciprocal plot against RNA concentration. The line shown is the best straight line through these points. The single black triangle shows the position on this graph of the grain counts after in situ hybridization of polyadenylated RNA isolated from the same preparation of polysomal RNA. Only one RNA concentration was used in this case. The RNA species complementary to loci 2-32A, 2-36A and 2-48BC are more concentrated in the polyadenylated RNA that in total polysomal RNA and we thus conclude that these RNAs are polyadenylated as are the analogous sequences in *D. melanogaster* (McKenzie et al., 1975; Spradling et al., 1975; Spradling et al., 1977).

Cytoplasmic 2-48BC RNA in Salivary Glands

Since the data showing that the puff at locus 2-48BC contains non-polyadenylated 40S RNA (Bisseling et al., 1976) were (obviously) obtained using salivary glands, it was of interest to determine whether a smaller, polyadenylated, 2-48BC RNA was present in the cytoplasm of this tissue also. For this purpose we used glands that had been maintained for 20 h in the medium and for 4 h in the same medium covered with liquid paraffin or for 60 min in the medium saturated with N_2. The total transcriptional activity of such glands in fresh

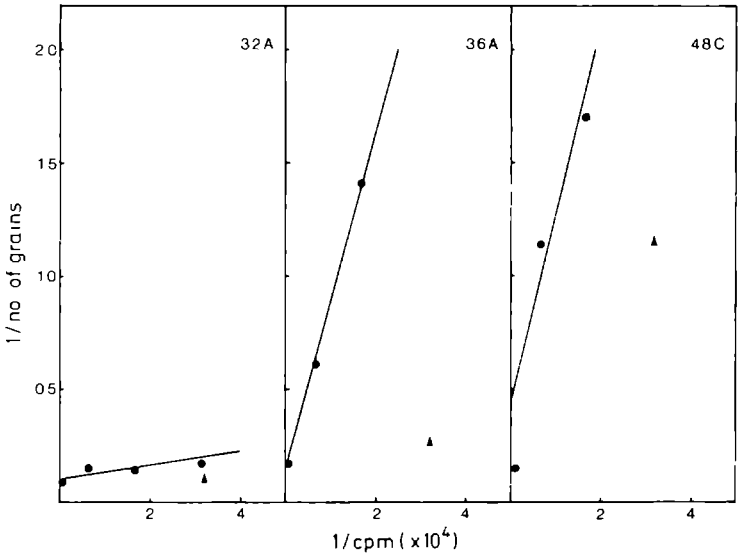

Fig 5. The presence of poly A in polysomal RNA. Polysomal RNA (6×10^4 cpm µg) was used for in situ hybridization at various concentrations ranging from 9×10^4 to 6×10^3 cpm µl. The extent of hybridization over locus 2-32A (33 nuclei total), loci 2-36A (24 nuclei total) and 2-48C (30 nuclei total) was quantitated (● ●). The data points shown are the average grain counts from the various slides. The parameters of these lines as calculated by the least squares method using each grain count as a single data point are: 2-32A slope $0.30 + 0.11 \times 10^3$ cpm µl^{-1}, intercept 0.11 ± 0.01; 2-36A slope $9.79 \pm 1.82 \times 10^3$ cpm µl^{-1}, intercept 0.16 ± 0.17; and 2-48C slope $12.8 \pm 3.5 \times 10^3$ cpm µl^{-1}, intercept 0.44 ± 0.34. Poly-A containing RNA was isolated from this RNA preparation as described in Methods and hybridized in situ at a concentration of 3.1×10^3 cpm µl and the amount of hybridization to loci 2-32A (20 nuclei counted), 2-36A (19 nuclei counted) and 2-48BC (16 nuclei counted) was again quantitated (▲)

medium was significantly reduced, but the regions which can be induced by a temperature treatment or when the glands recover from anaerobiosis, incorporate labelled precursors at a high rate (Fig. 6a). Autoradiographs of polytene chromosomes and gel electrophoresis of labelled RNA isolated from these glands indicated that the nucleolus incorporated labelled precursors very weakly compared to glands pulse labelled for 15 min after 24 h in vitro incubation in Poels medium (Fig. 6b).

Since the "heat-shock" sensitive loci are highly active in transcription, most of the cytoplasmic RNA labelled under these conditions should originate from these loci. An analysis via in situ hybridization of the labelled sequences present in total cytoplasmic RNA showed a light random distribution of grains over a variety of chromosome loci with, surprisingly, no specific increase over regions 2-32A, 2-36A and 4-81B, while region 2-48BC was clearly labelled. The apparent absence of sequences complementary to loci 2-32A, 2-36A and 4-81B may be due to a low concentration of these sequences in the hybridization mixture. The high concentration of labelled RNA complementary to locus 2-48BC, however, makes this material very suitable for a further investigation of the properties of this RNA.

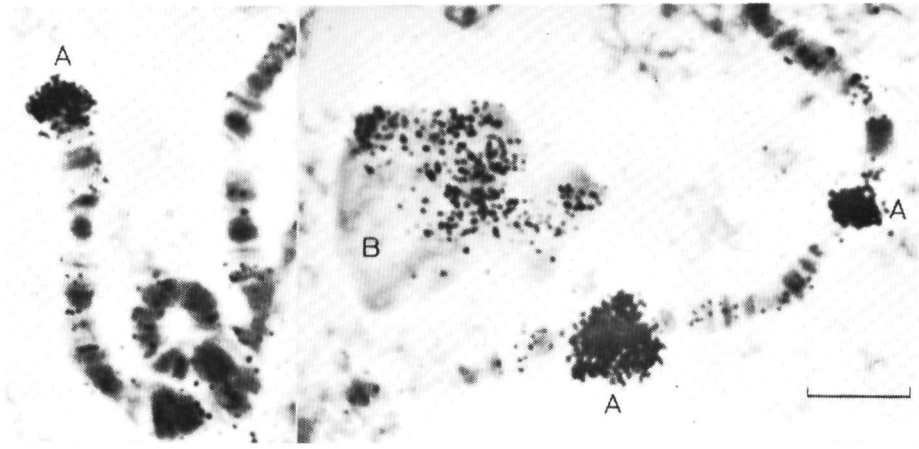

Fig. 6. Autoradiogram of pulse labelled salivary gland chromosomes. Salivary glands were pulse labelled for 15 min with ^{3}H-uridine after release from anaerobiosis as described in Methods. Exposure time was 10 d. *A* Heat-shock sensitive loci, *B* nucleolus

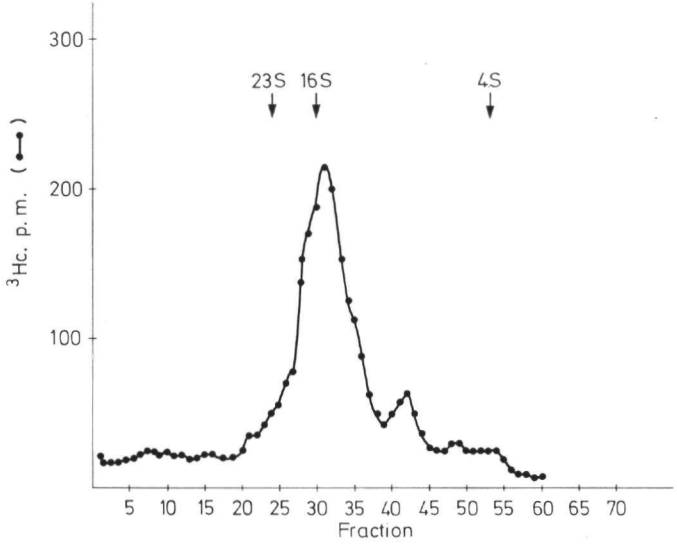

Fig. 7. Electropherogram of poly adenylated RNA. Poly-adenylated RNA was extracted from salivary glands labelled with radioactive RNA precursors after release from anaerobiosis

The poly-A containing species were isolated from the total cytoplasmic RNA by Poly-U-Sepharose chromatography. The column bound about 30% of the labelled cytoplasmic RNA and most of the eluted material migrated during gel electrophoresis with a size of about 15S (Fig. 7). After in situ hybridization of this RNA, only region 2-48BC was specifically labelled (Fig. 8). The properties

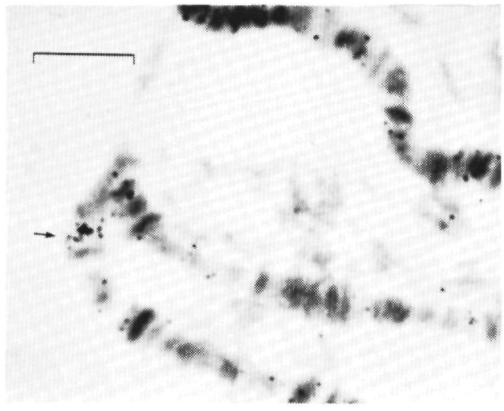

Fig. 8. In situ hybridization to locus 2-48BC. Poly-adenylated RNA, extracted from salivary glands labelled with radioactive RNA precursors after release from anaerobiosis, was hybridized in situ at a concentration of 7×10^3 cpm/μl. Exposure time was 90 d. The chromosome locus shown is 2-48BC

of cytoplasmic 2-48BC RNA from salivary glands appear thus to be very similar to those of such RNA from diploid cells.

Discussion

Previous experiments have shown that both during vitamin B_6 and temperature induction a non-poly-adenylated 40S RNA is found in the puff at locus 2-48BC (Bisseling et al., 1975). Salivary glands apparently also contain a small (3–4S) RNA species complementary to this locus (Berendes et al., 1973). On the other hand, Leenders et al. (1973) have found a correlation between the transcriptional activity of locus 2-48BC and increases in tyrosine transaminase activity and suggested that this locus might code for this enzyme or a subunit thereof. This suggestion requires the production of functional mRNA by this locus. Therefore, in the experiments reported here, we have been primarily concerned with the function and fate of the RNA originating from locus 2-48BC. The materials in the puff could be analyzed directly but in other cellular compartments, and in diploid cells, RNA from this locus can only be detected via in situ hybridization. With this method we have shown that polyadenylated RNA complementary to locus 2-48BC is found in the cytoplasm of salivary glands and is associated with the polysomes in diploid cells. Similarly, the other three major temperature puffs of *D. hydei*, 2-32A, 2-36A and 4-81B (data for this locus are not shown here) also code for RNA that has all the characteristics of functional mRNA.

The association of 2-48BC RNA with polysomal material must be specific and cannot be due to co-sedimenting material that has leaked out of the nuclei during the isolation procedure since the hybridization characteristics of nuclear

RNA complementary to locus 2-48BC differ markedly from those of cytoplasmic RNA: nuclear RNA hybridizes to more DNA at locus 2-48BC than polysomal RNA does. If we assume that the specific activities of the nuclear and polysomal 2-48BC RNA are about equal, then nuclear RNA hybridizes to about five times as much DNA as polysomal 2-48BC RNA does. In this respect 2-48BC RNA differs from either 2-32A or 2-36A RNA. In these cases no nuclear RNA larger than polysomal RNA was detected within the limits of the accuracy of these experiments.

The finding of a large nuclear RNA complementary to locus 2-48BC is not unexpected since Bisseling et al. (1976) have shown that a 40S RNA is present in the puff at this locus in salivary glands. They also occasionally found a 16S RNA at this site. Since the 16S RNA was present in varying and small amounts relative to the amount of 40S RNA found, they concluded that the 40S RNA was the major transcription product and that there was probably no precursor-product relationship between the two species. Nevertheless, the 40S RNA could be destructively processed outside the puff area to yield the smaller cytoplasmic 2-48BC RNA. Alternatively, the 16S RNA also found in the puff could be the direct precursor to the cytoplasmic RNA while the 40S RNA would then turn over inside the nucleus. In either case two genes would be active at this site. We cannot distinguish between these possibilities since we have as yet been unable to extract large nuclear RNA species from tissue culture cells, presumably due to the high level of RNase in these cells. Experiments designed to overcome this technical problem are in progress. At this time we have no explanation for the small (3–4S) RNA complementary to locus 2-48BC found in salivary glands by Berendes et al. (1973). If the small size of this RNA is not due to RNase action during its isolation, then this finding would suggest that locus 2-48BC may also have a repetitive element since a unique RNA of that size would not have been detected under their experimental conditions.

If we assume that the specific activities of the various RNA species complementary to the "heat-shock" loci extracted from tissue culture cells are about equal, then, from the maximum grain densities at saturating RNA concentrations, locus 2-32A codes for about twice as much RNA as locus 2-36A, the product of which is about twice as long as the cytoplasmic 2-48BC RNA. The size of the polysomal RNA complementary to locus 2-36A is approximately 20 (Sondermeijer and Lubsen, in prep.), while locus 2-32A is often seen to be double in autoradiographs of in situ hybridization and the puff at this site in polytene chromosomes is due to the activation of two bands, 2-31C and 2-32A (Berendes et al., 1965). Thus this region probably codes for two RNA species.

Furthermore, in our autoradiographs of in situ hybridization, the grains are located just to the right of region 2-48B3 over the part of the region with three thin bands (2-48B4-5-6), suggesting that the coding sequence for the "heat-shock" locus is in this region. This is in agreement with the observations of Berendes et al. (1973), who found RNP accumulation in the region of bands B_4 and B_5 and the interband in between 10 to 20 min after the onset of puff induction.

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Note Added in Proof

Recently S Henikoff and M Meselson (Cell **12** 441 451 1977) have shown that the heat-shock sensitive locus 87C in *Drosophila melanogaster* also appears to produce additional nuclear RNA sequences

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CHAPTER IV

The Products of the "Heat-Shock" Loci of *Drosophila hydei*

Correlation between Locus 2-36A and the 70,000 MW "Heat-Shock" Peptide

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Abstract. Antibody was raised against total *Drosophila hydei* embryonic cellular protein with a molecular weight between 65,000 and 70,000 dalton. This antiserum reacted with the 70,000 MW "heat-shock" peptide found in ^{35}S labelled cell extracts of "heat-shocked" *D. hydei* tissue culture cells or salivary glands. – The antibody was coupled to Sepharose 4B and this material was used to absorb polysomes obtained from tissue culture cells incubated at 37° C in the presence of tritiated RNA precursors. The relative concentrations of various RNA species complementary to the "heat-shock" loci 2-32A, 2-36A, and 2-48C in either bound, non-bound, or total polysomal material was then determined by in situ hybridization. The RNA species complementary to locus 2-36A was found to be enriched in the bound polysomal material.

Introduction

The experimental activation of the so-called "heat shock" loci in *Drosophila* is accompanied by a change in protein synthetic pattern, namely the appearance of six, new, polypeptide bands (Tissieres et al., 1975; Lewis et al., 1975; Koninkx, 1976). These peptides are always found in the protein synthetic pattern of salivary glands which have been exposed to various treatments such as a release from anaerobiosis or exposure to arsenite, all of which induce the same set of puffs. These peptides are especially marked during incubation of cells at 37° C, since during this "heat shock" the normal synthetic pattern is largely suppressed (Koninkx, 1976). Diploid cells, such as tissue culture cells, respond in the same manner as salivary gland cells to a "heat shock", since qualitatively the same set of "heat shock" peptides appears and RNA can be isolated from these tissues that hybridizes back in situ to the "heat shock" sensitive sites on salivary gland chromosomes (McKenzie et al., 1975; Spradling et al., 1975, 1977; Lubsen et al., 1977).

The suggestion has been made that the "heat shock" sensitive loci provide the mRNA species which code for these "heat shock" peptides. These loci do indeed code for mRNA since sequences complementary to these loci are found polyadenylated and associated with the polysomes, both in *D. melanogaster* (McKenzie et al., 1975; Spradling et al., 1975, 1977) and in *D. hydei* (Lubsen et al., 1977). However, the coding properties of these mRNA species or the specific gene loci for the "heat shock" proteins have as yet not been determined.

The gene coding for a particular protein can be identified in a variety of ways. One possibility is to purify the polysomes synthesizing this protein immunochemically (see for example Schimke et al., 1974). The chromosomal locus can then be determined via in situ hybridization of the mRNA present in these isolated polysomes. We have used this procedure to show that polysomes, which are retained by a column of Sepharose coupled to antibody to the 70,000 dalton "heat shock" peptide, are enriched for RNA complementary to locus 2-36A of *D. hydei*.

Materials and Methods

Conditions for Labeling of Cells, Isolation of Polysomes and ^{35}S Labelled Extracts. Cells were either obtained as five day old primary cultures from *D. hydei* embryos established as described (Shields and Sang, 1970; Lubsen et al., 1977) or a *D. hydei* cell line established in our laboratory (Sondermeijer and Lubsen, in prep.) was used.

Polysomes were prepared from cells incubated with tritiated RNA precursors for 1 h at 37°C as described (McKenzie et al., 1975; Lubsen et al., 1977). For ^{35}S methionine labeling the cell medium was replaced with medium without added methionine 16 h before the labeling period. Cells were preincubated for 45 min at 37°C. 10 μC/ml ^{35}S methionine was added and incubation was continued for 15 min. Cells were collected by centrifugation, washed with insect Ringer and dissolved in two volumes of 8 M urea, 0.2% SDS. Such extracts were passed over a G-25 Sephadex column and before immunoaffinity chromatography over a Sepharose 4B column equilibrated with immunoprecipitation buffer (see below).

Immunoaffinity Chromatography. Cells were prepared from 10–12 h old *D. hydei* embryos after a 2 h heat-shock as described (Shields and Sang, 1970; Wiltenburg and Lubsen, 1976). The cell pellet was dissolved in twice its volume SDS sample buffer and the resulting extract was subjected to SDS-gel electrophoresis on 3 mm thick slab gels as described (Koninkx, 1976). The appropriate MW region was detected by staining with Coomassie blue and cut out. The polyacrylamide strips obtained from three such gels were pooled, homogenized in saline and the homogenate was injected into rabbits according to the procedure described by Tjian et al. (1975) except that no Freund's adjuvant was added. Double antibody precipitation tests to detect antibody were performed as described by Gielkens et al. (1976) using a ^{35}S labelled extract from heat shocked cells as a source of antigen. Immunoglobulin was partially purified and freed of RNase activity as described by Schimke et al. (1974) and coupled to CNBr activated Sepharose 4B as described by Livingstone (1974). Immuno absorbant chromatography was performed under the conditions described by Schimke et al. (1974). Polysomes (75 μg) were resuspended in 10 mM Tris pH 7.2, 150 mM NaCl, 5 mM $MgCl_2$, 1% Triton X100 and 0.1 mg/ml heparin (TMN buffer) and loaded on a 0.5 ml column which was equilibrated with the same buffer containing 1 mg/ml BSA. The column was washed with two volumes of TNM buffer containing 0.5 M sucrose, then with two volumes of TNM buffer without detergent and the RNA was eluted with five column volumes 10 mM Tris pH 7.5, 0.5 mM EDTA and 0.1 mg/ml heparin. The eluted material was collected in five equal fractions and the eluted RNA was usually present in the first three. The column was regenerated by washing with six column volumes of 0.1 M NH_4OH, 0.1% SDS and reequilibrated. The unbound fraction was then rechromatographed over the same column. All steps were performed at 4°C.

Polysomes not bound to the column were collected by centrifugation while the material bound to and eluted from the column was collected by alcohol precipitation. RNA was purified by phenol chloroform extraction and repeatedly washed with 3 M NaAc pH 6. The RNA was finally dissolved in a small volume of 2 × SSC and used for in situ hybridization.

Immunoaffinity chromatography of ^{35}S labelled cell extract was performed in the same manner except that the column was eluted directly with 0.1 M NH_4OH 0.1% SDS.

In situ Hybridization. In situ hybridization was performed essentially as described by Pardue and Gall (1975) and by Lubsen et al. (1977). Exposure times varied from 14 to 60 d depending upon the amount of RNA applied. For the quantitation of the patterns, grains were counted over the appropriate chromosomal locus and the grain count was corrected to a two week exposure time. Grain counts were then plotted in a double-reciprocal plot against RNA concentration and the best straight line through these points was calculated by the least-squares method. The number of nuclei counted per slide varied with the quality of the squash and with the chromosome morphology but averaged about 10 per slide.

Materials. Sepharose 4B-CNBr was obtained from Pharmacia. DEAE- and CM-cellulose were from Whatman. Goat-anti-rabbit-γ-globulin was from Calbiochem. ^3H-uridine (46 Ci/mM), ^3H-guanosine (10 Ci/mM) and ^3H-cytosine (28 Ci/mM) were from the Radiochemical Centre, Amersham while ^3H-adenosine (33 Ci/mM) was obtained from New England Nuclear. All other chemicals used were reagent grade.

Results

Since purified "heat-shock" peptides are not available, we have raised antibodies against cellular proteins with a molecular weight between approximately 65,000 and 75,000 daltons (Fig. 1). This procedure yielded antiserum which reacted with polypeptides labelled during incubation at 37°C as assayed by double immunoprecipitation. Analysis of the double immunoprecipitate by SDS poly-

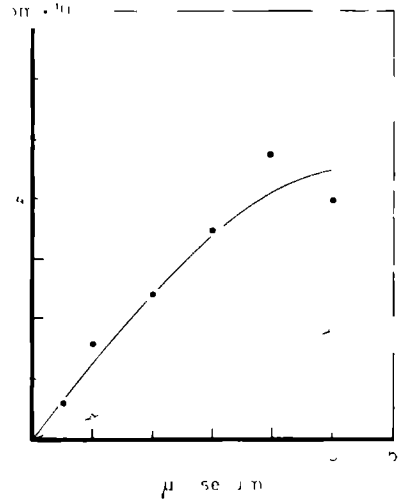

Fig. 1. Double immunoprecipitation of ^{35}S labelled proteins from extracts of heat shocked cells. ^{35}S labelled cell extracts (1.2×10^4 cpm) were incubated with various amounts of rabbit antiserum and subsequently with goat-anti-rabbit serum as described in Methods. The immunoprecipitate was dissolved in SDS buffer and counted in a toluene-triton X-100 based scintillation fluid.
● ● immune serum
○ ○ control serum

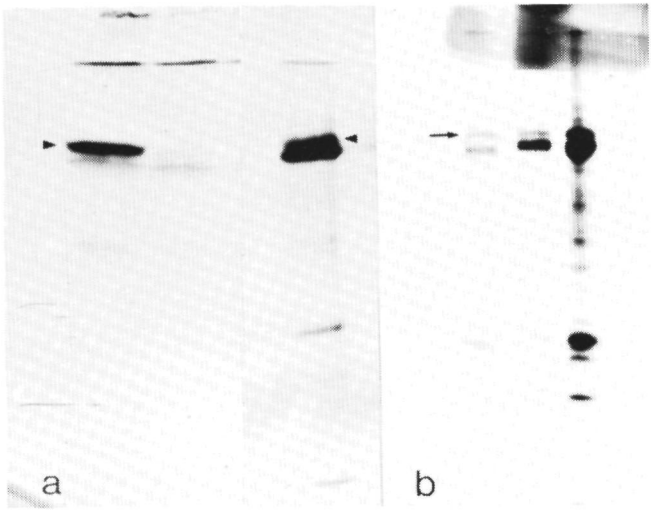

Fig. 2a and b. Autoradiograph of SDS gel electrophoresis pattern of ^{35}S labelled proteins bound by antiserum. **a** ^{35}S labelled proteins were precipitated from extracts of heat-shocked salivary glands by double immunoprecipitation as described in Methods. From right to left: ^{35}S labelled peptide pattern of salivary gland extract, ^{35}S labelled peptide pattern of material precipitated by control serum and ^{35}S labelled peptide pattern of material precipitated by immune serum. **b** ^{35}S labelled extracts of heat-shocked cells were passed over an immunoaffinity column as described in Methods. From right to left: ^{35}S labelled peptide pattern of the cell extract, ^{35}S labelled peptide pattern of material not bound to the column and ^{35}S labelled peptide pattern of material bound to and eluted from the column. The arrows point to the 70,000 MW "heat-shock" band

acrylamide gel electrophoresis and autoradiography showed that only a single labelled peptide, corresponding to the 70,000 MW "heat shock" peptide, is bound by the antiserum (Fig. 2a). The same specificity for the 70,000 MW "heat shock" band is shown by an immunoaffinity column prepared from this antiserum, although the background is somewhat higher (Fig. 2b). This higher background could be due not only to unspecific binding but also to incomplete elution of the affinity column: usually only about fifty procent of the bound ^{35}S labelled material can be eluted. The high affinity antibody-antigen complexes may resist dissociation and the amount of antigen actually bound would then be underestimated in the eluted fraction.

Analyses of the protein synthetic pattern of tissue culture cells incubated at 37° C by two dimensional electrophoresis have shown that the 70,000 MW band contains only a single peptide and that the heat-shock peptide is the only peptide with this molecular weight synthesized by the cells at 37° C (Sondermeijer and Lubsen, in prep.). The antibody is thus monospecific with respect to the "heat-shock" proteins. With the aid of this antiserum we have tried to specifically isolate polysomes coding for the 70,000 MW peptide. Usually polysomes are isolated immunochemically either via direct immunoprecipitation or via double immunoprecipitation (see for example Schimke et al., 1974). We have chosen to prepare instead an immunoaffinity column by coupling partially purified immunoglobulin to Sepharose 4B.

As starting material for the specific isolation of polysomes containing the 70.000 MW peptide nascent chains, ^{3}H-labelled polysomes isolated from tissue culture cells incubated for one hour at 37° C were used. At 37° C, these cells synthesize virtually exclusively the "heat shock" polypeptides and the 70.000 MW polypeptide accounts for about 10% of total protein synthesis (Fig 2b) Radioactive RNA precursors were added only during the incubation at 37° C This procedure presupposes to a certain extent that the RNA species one attempts to purify is indeed encoded by a "heat-shock" locus, since only the products of the "heat-shock" loci, which account for most of the RNA synthesis at 37° C (Spradling et al., 1977), have a high enough specific activity to be detected by in situ hybridization We at first attempted to avoid this fallacy by either adding radioactive precursors at 12 h intervals starting 48 h before the "heat-shock" or by iodinating polysomal RNA Both methods, however, yielded such a heavy general chromosomal labeling after in situ hybridization that it was impossible to analyse the grain density over any one locus For ease of interpretation then, it was necessary to use polysomes labelled in vivo at 37° C

When such polysomal material is passed over an immunoaffinity column typically about 25% of the radioactivity remained bound to the column About half of this material could be eluted with high concentrations of EDTA. Subsequent elution with NH_4OH and SDS, which also eluted the polypeptide chains, removed about half of the remainder. In general the yield of "purified" material was about 10% of the starting material. The material eluted with EDTA was de-proteinized, collected by ethanol precipitation and used for in situ hybridization. Its in situ hybridization pattern was then compared with that of total polysomal RNA or of non column bound RNA

We have previously shown that only the three "heat-shock" loci on the second chromosome (2-32A, 2-36A and 2-48BC) and, occasionally, a non "heat-shock" locus, 3-59A contain a significant amount of label when polysomal RNA isolated from cells incubated at 37° C in the presence of tritiated RNA precursors is used for in situ hybridization. The grain density over the fourth "heat-shock" locus (4-81B) is only rarely above background (Lubsen et al., 1977) Locus 3-59A is also prominent if RNA from cells labelled at 25° C is used for in situ hybridization and is apparently always active in cultured *D hydei* cells An example of in situ hybridization to loci 2-32A and 2-36A using either column bound or unbound RNA is shown in Figure 3 We have analyzed quantitatively only the grain counts over loci 2-32A, 2-36A, 2-48BC and, when present, 3-59A An example of such grain counts is shown in Table 1 in this experiment the in situ hybridization pattern of column bound or flow through polysomal RNA at various concentrations was determined These data indicate a stronger labeling of locus 2-36A by column bound RNA but do not allow a direct calculation of such an enrichment since the grain counts are not directly proportional to RNA concentration We have therefore made a double reciprocal plot of the numbers of grains over a particular chromosomal locus as a measure of the hybridization percentage against the cpm/μl applied as a measure of the RNA concentration. This procedure yields a straight line when applied to data obtained by filter hybridization (Bishop, 1969). The slope of this line

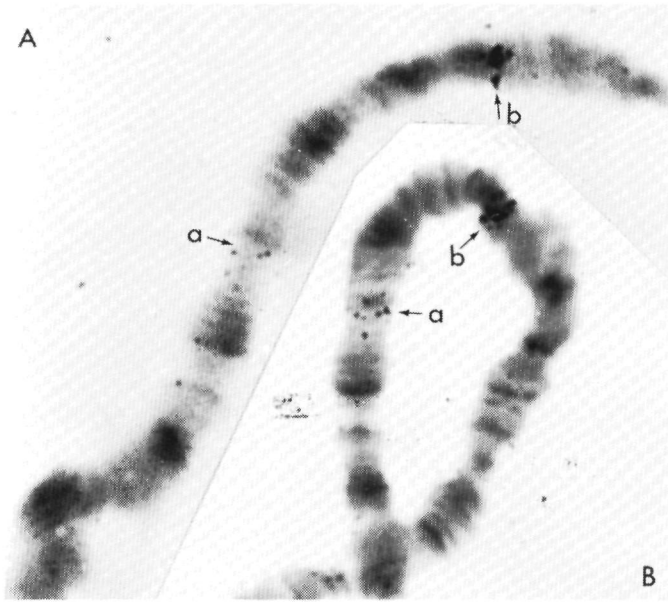

Fig. 3A and B. In situ hybridization of total polysomal and column purified polysomal RNA. Polysomes (6×10^4 cpm/μg) obtained from primary cultures were purified by immunoaffinity chromatography as described in Methods. The in situ hybridization pattern of the starting material (total polysomal material) and the column-bound material was then compared. **A** Unbound polysomal RNA (1.5×10^4 cpm/μl). **B** Column bound RNA (1.4×10^4 cpm/μl). Exposure time was 30 d. The "heat-shock" sensitive loci are indicated as follows: *a* 2-36A, *b* 2-32A

Table 1. Grain densities over three "heat shock" loci after in situ hybridization of either column-bound of flow-through polysomal RNA. Polysomal RNA, obtained from "heat-shocked" primary cultures, was hybridized in situ at the concentrations indicated as described in Methods. n: Number of chromosomal loci counted. S.E. standard deviation. Exposure time was 30 d. The individual grain counts obtained in this experiment, but corrected to an exposure time of 14 d, were used to calculate the parameters of the lines presented as the results of experiment 1 in Table 2

	RNA conc. (cpm/μl)	No. of grains ± S.E. in n nuclei of locus					
		n	32A	n	36A	n	48BC
Flow through polysomal RNA	9.1×10^4	10	27.8 ± 10.3	7	12.7 ± 3.2	10	14.4 ± 3.5
	1.5×10^4	12	14.6 ± 4.1	11	3.5 ± 2.4	12	1.9 ± 1.8
	0.6×10^4	11	15.5 ± 3.7	6	1.5 ± 0.8	8	1.2 ± 0.7
Bound polysomal RNA	1.4×10^4	21	19.0 ± 5.9	21	6.9 ± 2.8	18	2.9 ± 2.1
	0.6×10^4	10	13.4 ± 6.1	10	3.7 ± 1.3	8	0.1 ± 0.4
	0.3×10^4	19	9.9 ± 2.2	18	2.4 ± 1.3	18	0.8 ± 1.0

is a measure of the concentration of the specific RNA species relative to the total labelled RNA concentration. If the complexity and the specific activity of the RNA is held constant, which is the case if one compares subsets of one polysome preparation, the relative concentration of RNA complementary to any one locus in these subsets can then be determined.

The results of two typical experiments are shown graphically in Figure 4,

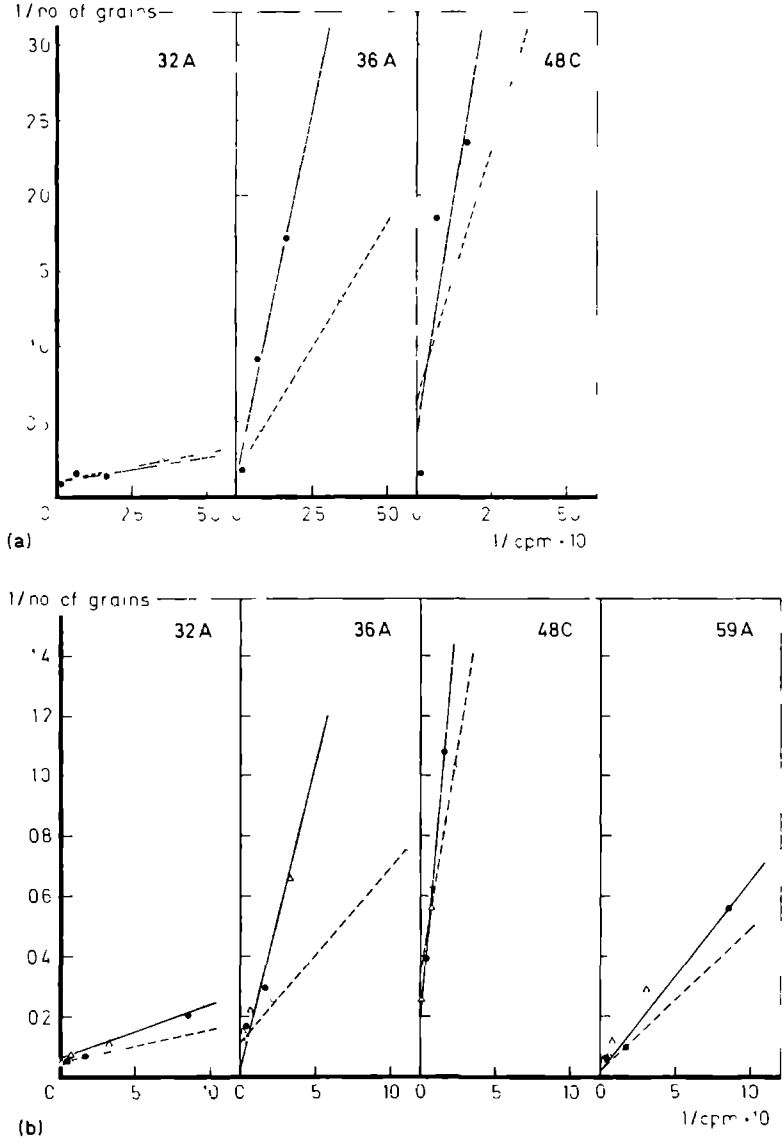

Fig. 4a and b. Concentration dependency of in situ hybrid formation of polysomal RNA isolated by immunoaffinity chromatography **a** A primary culture of *D. hydei* embryonic cells was incubated with 10 μC ml of ^{3}H-uridine for 48 h then cells were shifted to 37°C and more tritiated RNA precursors were added as described in Methods Polysomes were isolated and separated into two fractions material that did and material that did not bind to the column RNA (6×10^4 cpm/μg) isolated from these two fractions was hybridized in situ at concentrations ranging from 9×10^4 to 6×10^3 cpm/μl (unbound material) or 1.4×10^4 to 3×10^3 cpm μl (bound material) The hybridization to loci 2-32A 2-36A and 2-48BC was quantitated ● ● unbound polysomal RNA ○---○ bound polysomal RNA The data points shown are the average grain density of the various slides **b** Polysomes were isolated from *D. hydei* cultured cells after incubation and labeling as described in Methods The in situ hybridization of total polysomal RNA was compared with that of RNA obtained from polysomal material retained by and eluted from immunoaffinity column RNA (6.3×10^4 cpm/μg) was used at concentrations ranging from 3.0×10^4 to 1.2×10^3 cpm/μl total polysomal RNA and 2.3×10^4 to 9.0×10^2 cpm/μl (bound polysomal RNA) Hybridization to loci 2-32A, 2-36A 2-48BC and 3-59A was quantitated ● ● total polysomal RNA ○ - ○ bound polysomal RNA unbound RNA The data points shown are the average grain density of duplicate slides

Table 2. Parameters of the concentration dependency of in situ hybrid formation of polysomal RNA. The parameters of these lines were calculated as described in Methods. Conditions of in situ hybridization are given for experiment 1 in the legend to Figure 4a and for experiment 2 in the legend to Figure 4b. Note that the cells from which the material used in experiment 1 was obtained, were prelabelled for 48 h. Therefore the specific activity of the mRNA obtained from these cells is lower than that used in experiment 2 eventhough the specific activities of the polysomal RNA are about equal, and the intercept obtained in experiment 1 is thus higher than in experiment 2. n: Number of nuclei counted. No ± SE: number of grains ± standard error.

RNA	Locus	n	Intercept grains $^{-1}$ 10^{-3}	Slope (cpm grains $\times 10^{3}$)	RNA concentration relative to unbound or total polysomal RNA
Exp. 1					
Flow through polysomal	32A	33	1.1 ± 0.1	0.30 ± 0.11	
	36A	24	1.6 ± 1.7	9.79 ± 1.82	
	48C	30	4.4 ± 3.4	12.8 ± 3.5	
Column bound polysomal	32A	50	1.0 ± 0.1	0.37 ± 0.06	0.8
	36A	49	1.9 ± 2.4	3.28 ± 1.04	3.0
	48C	36	6.5 ± 2.8	6.86 ± 1.18	1.9
Exp. 2					
Total polysomal	32A	58	0.65 ± 0.19	0.17 ± 0.04	–
	36A	55	0.40 ± 1.2	1.99 ± 0.26	
	48C	36	2.0 ± 2.0	5.75 ± 1.57	–
	59A	38	0.18 ± 0.21	0.63 ± 0.06	–
Flow through polysomal	32A	46	0.60 ± 0.07	0.17 ± 0.03	1
	36A	40	1.10 ± 0.86	1.76 ± 0.42	1.1
	48C	25	1.84 ± 2.26	5.74 ± 4.05	1
	59A	26	0.60 ± 0.24	0.73 ± 0.11	0.9
Column bound polysomal	32A	47	0.47 ± 0.07	0.11 ± 0.01	1.5
	36A	51	1.14 ± 0.3	0.59 ± 0.06	3.4
	48C	30	3.38 ± 1.4	3.16 ± 0.88	1.8
	59A	34	0.25 ± 0.17	0.47 ± 0.03	1.3

while the parameters with their standard deviation of the lines and the calculated RNA concentrations relative to unbound or total polysomal RNA are given in Table 2. The data in experiment 1 (Fig. 4a) were obtained using polysomes from cells in primary culture and the hybridization patterns of the flow through and column-bound polysomal RNA were compared. The bound fraction is enriched for RNA complimentary to locus 2-36A, slightly depleted for RNA complementary to locus 2-32A while the concentration of material complimentary to locus 2-48BC is approximately the same in both preparations. Experiment 2 is similar, but polysomes isolated from a cell line were used and the in situ hybridization pattern of the starting material (total polysomes), the pattern of the flow through material, and the pattern obtained from the polysomes bound by the immunoaffinity column were compared (Fig. 4b). Again,

the bound materials is clearly enriched for material complimentary to locus 2-36A but also appears to be enriched, although to a lesser extent for sequences complimentary to the other loci analysed. This low level enrichment is probably an artifact: the data obtained for locus 3-59A suggest that the RNA from the bound preparation hybridized slightly more efficiently than the RNA from the total polysomes. Indeed, in this experiment locus 3-59A serves as a control for such an effect since 3-59A RNA is also present in high concentration in polysomal material isolated from cells labelled at 25°C and cannot code for the 70,000 MW polypeptide which is not detectably synthesized at 25°C.

In contrast to the experiments reported above, no enrichment of 2-36A sequences was found in "heat-shocked" polysomal RNA bound to and eluted from an immunoaffinity column prepared from antiserum against partially purified *D. hydei* mitochondrial NADH dehydrogenase (L. Hermans, unpublished observations).

Discussion

The interpretation of the data presented above is critically dependent upon, first, the specificity of the antiserum used to purify polysomes, and secondly, the reliability of the in situ hybridization data. Since our antiserum was raised against all cellular proteins with a molecular weight between 65,000 and 75,000, our antiserum might be expected to react with both the 67,000 and the 70,000 MW "heat-shock" peptides. However, our antiserum reacts only with the 70,000 MW band. One can only hope to raise antibody against a "heat-shock" peptide if this peptide is normally present in the cell, since if it is made only when the "heat-shock" puffs are active, its concentration in the isolated protein mixture will probably be too low to be antigenic. One might reasonably expect that a "heat-shock" peptide which also plays a role in the normal cellular metabolism, would persist in the cell while "heat-shock" peptides which are required only in response to the imposed metabolic stress might be expected to be broken down when the metabolic stress is alleviated. Of the two high MW bands (67,000 and 70,000 MW) which are labelled at 37°C, only the 70,000 MW band is stable during subsequent incubation of the cells at 25°C while the 67,000 MW band disappears with a $T_{1/2}$ of approximately 3 h (unpublished data). The instability of the 67,000 MW peptide could explain its lack of antigenicity. One might furthermore expect the antiserum to react with other, non "heat-shock", proteins of this molecular weight present in the cell extract. However, such a cross-reaction should not interfere with our analysis, since the procedure relies on the purification of polysomes via the interaction of the antibody with nascent chains and since "heat shocked" cells synthesize primarily "heat-shock" peptides.

To allow conclusions regarding relative RNA concentrations from in situ hybridization patterns, it is necessary to analyse these quantitatively. It is clear from Table 1 that such a quantitative analysis suffers from a rather high degree of uncertainty. This error apparently arises from the scatter of values obtained for different nuclei on one single slide, since, if the average grain count from

one slide is used to calculate the line instead of each single grain count, the fit of the data is much better. This problem is thus inherent in the methodology used, especially when grain counts are low. Nevertheless, the differences in concentration of 2-36A RNA between column-bound RNA and total polysomal RNA or unbound RNA are statistically highly significant ($p \leqq 0.005$). In principle, in situ hybridization can be used for the detection and quantitation of any RNA sequence. In practice, only RNA sequences present in relatively high concentrations and, of course, with a high specific activity, are detected. Furthermore, as we have already stated above, to allow a quantitative analysis, it is necessary to be even more selective and to use RNA preparations which contain only a few detectable separate components. The addition of radioactive precursors only during "heat-shock" yields polysomal RNA preparations which meet these criteria, but, as also pointed out above, such a labeling protocol assumes inherently that the product of one of the "heat-shock" loci will be the one sought. *D. hydei* has four major heat shock sensitive loci (Berendes et al., 1965): the three on the second chromosome have been analyzed quantitatively in this study. In situ hybridization to the fourth locus, 4-81B, is difficult to quantitate since the grain density over this locus is very low.

However, preliminary data showed no significant enrichment of this RNA after immunoaffinity column chromatography. Furthermore, we have not analyzed hybridization over any non "heat shock" loci except locus 3-59A, which is the only prominently labelled non "heat shock" locus under these conditions. Therefore, we cannot rule out the possibility that, after immunoaffinity chromatography, RNA complimentary to a non "heat-shock" sensitive locus could have been enriched more than 2-36A RNA. However, if the "heat-shock" loci code for the "heat-shock" peptides, as indicated by the actinomycin D sensitivity of their synthesis (Lewis et al., 1975, McKenzie et al., 1975, Koninkx, 1976), then locus 2-36A is likely to code for the 70,000 MW heat shock peptide in *D. hydei*.

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CHAPTER V

Heat-Shock Peptides in *Drosophila hydei* and Their Synthesis *in vitro*

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The protein-synthetic response to a heat shock, namely the synthesis of a number of new peptides, of *Drosophila hydei* salivary glands and tissue-culture cells appears qualitatively the same when analyzed by dodecylsulphate electrophoresis, except that in tissue-culture cells an additional band at 25500 M_r appears while the 20000-M_r peptide, found in salivary glands, is sometimes absent depending on the cell line used. Further analysis of the heat-shock peptides by two-dimensional electrophoresis showed that the response of salivary glands and embryonic cells also differs: two major proteins with a molecular weight around 26000 were found in embryos, one of which may be identical to the 25500-M_r protein seen in cultured cells. Two-dimensional heat-shock patterns are further characterized by a number of minor polypeptides whose position and intensity vary slightly from one tissue to another.

Polysomal poly(A)-containing RNA extracted from heat-shocked embryos and tissue-culture cells directed the synthesis in a wheat germ extract of all heat-shock peptides found *in vivo* in the same tissue. Therefore, the appearance of one additional protein in embryos and tissue-culture cells does not arise from tissue-specific post-translational modifications but must be due to the induction of an additional gene as compared to salivary glands. A similar conclusion may hold for the minor heat-shock proteins found in the two-dimensional patterns *in vivo* as well as *in vitro*.

The translation of mRNAs *in vitro* has proved to be a useful method in analyzing one of the essential properties of such RNA, namely the protein chain it codes for. A number of cell-free systems are known which are able to translate heterologous mRNA species. These include, for instance, Krebs II ascite tumor cell extracts [1], reticulocyte lysates [2, 3] and wheat germ extracts [4]. Translation in wheat germ extracts is highly dependent upon the addition of RNA and we have chosen to use this system to study the synthesis *in vitro* of a set of newly synthesized polypeptides appearing after experimental gene induction in *Drosophila* by treatments which interfere with the normal cellular metabolism [5–7]. When such treatments, for example a heat shock or release from anaerobiosis, are applied to *Drosophila* cells, a number of loci (the heat-shock loci) are activated [8–13]. These active loci can be visualized on the salivary gland polytene chromosomes as puffs, while in diploid tissue-culture cells it has been shown that polyadenylated RNA species complementary to these loci are found associated with the polysomes [14–16]. The activation of the heat-shock loci is correlated with and probably causally related to the appearance of a number of new peptides in the protein synthetic patterns, since after all treatments that induce these loci qualitatively the same set of heat-shock peptides appears in salivary glands [5–7]. A similar set of bands also appears in tissue-culture cells after a heat shock, but in *D. hydei* cells some of these differ in properties (such as molecular weight and isoelectric point) from those found in salivary gland cells. We have therefore studied the translation *in vitro* of polysomal RNA, isolated from either heat-shocked tissue-culture cells or embryos, to determine to what extent these differences are related to the mRNA species present or due to possible post-translational processing of these peptides.

Abbreviation. Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

MATERIALS AND METHODS

Culture of Cells

Tissue-culture cell lines of *D. hydei*, recently established in our laboratory (Sondermeijer and Lubsen, unpublished results), were used. Cell lines grow in monolayers as well as in suspension or spinner cul-

tures at densities between 5×10^6 cells ml and 1×10^7 cells ml. Cells have a doubling time of about 30 h in Shields and Sang's revised medium [17].

[^{35}S]-Methionine-Labelled Protein Extracts

Salivary glands were hand isolated from late third instar larvae. Five pairs were incubated in Poels [18] or Shields and Sang's [17] medium for 60 min at 25 °C and 37 °C. Glands were transferred to 10 µl fresh medium and labelled at 25 °C for 20 min with 20 µCi [^{35}S]methionine (300 Ci mmol). After rinsing in Ringer's solution glands were dissolved in 50 µl lysis buffer (9.5 M urea, 0.5% Triton X-100, 5% 2-mercaptoethanol). Aliquots of 2 µl were used for dodecylsulphate electrophoresis while 5 µl was used for two dimensional electrophoresis.

Labelled protein extracts from *D. hydei* cells were prepared from monolayer cultures growing in 26.1 cm^2 Nunc flasks in Shields and Sang medium. Medium was replaced by medium without methionine 24 h before the onset of the experiments. After a 20 min preincubation at 25 °C or 37 °C, 10 µCi ml [^{35}S]methionine was added and incubation was continued for 60 min. Cells were collected and washed in Ringer's solution and dissolved in 25 µl of the same lysis buffer as used for glands. The amount of material used for gel electrophoresis was the same as that of salivary glands.

Purification of Poly(A)-Containing RNA

About 1.5 g embryos 0–15 h old were collected, dechorionated and incubated dry for an additional 90 min at 25 °C or 37 °C. After homogenization of the eggs and repeated washing of the cells in chilled homogenization medium [19] containing 100 µg ml cycloheximide, the embryonic cells were disrupted by vortexing for a few seconds in 2 ml 0.05 M Tris-HCl (pH 7.4), 0.25 M KCl, 0.05 M $MgCl_2$, 100 µg ml polyvinylsulfate and 0.5% Triton X-100. Cell debris was removed by centrifugation at $30000 \times g$ for 10 min, the supernatant was layered on sucrose gradients, centrifuged in an IEC SB 243 rotor for 72 min and polysomal regions were collected [14].

Tissue culture cells were submitted to a temperature treatment by warming up a 200 ml spinner culture containing 1×10^7 cells ml in a water bath to 37 °C over a 15-min period and keeping it at this temperature for an additional 45 min during which spinning was continued. Polysomes were isolated essentially as described by McKenzie et al. [14]. Polysomes were collected from the appropriate sucrose gradient fractions by 50% alcohol precipitation, dissolved in 2 ml of buffer A (0.01 M Tris-HCl, pH 7.4, 0.01 M EDTA, 0.5% dodecylsulphate) and heated for 2 min at 80 °C. The solution was made 0.5 M NaCl and applied directly to a poly(U)-Sepharose (Pharmacia) column. No more than 20 A_{260} units of polysomal RNA were applied ml gel volume. After repeated washes with buffer A + 0.1 M NaCl, poly(A)-containing RNA was eluted with three bed volumes 90% formamide, 10% buffer A (pH 7.2) at 65 °C. Fractions were alcohol precipitated and RNA was dissolved in H_2O at a concentration of 1.5 µg µl. This procedure yielded an RNA with high activity in the wheat germ translation assay. In contrast only poor results were obtained when polysomal RNA was submitted to phenol-chloroform extraction prior to chromatography on poly(U)-Sepharose. Sedimentation values of poly(A)-containing RNA were determined on 15–30% linear sucrose gradients in 0.01 M Tris-HCl (pH 7.3), 0.1 M NaCl and 0.5% dodecylsulphate by centrifuging for 4 h at 55000 rev/min in an IEC SB 408 rotor at 18 °C. 0.25 A_{260} unit of poly(A)-containing RNA was layered on each gradient and the size was determined relative to ribosomal RNA markers from *Escherichia coli* (BDH). Samples were heated for 2 min at 80 °C to break up aggregates.

Protein Synthesis in vitro

Crude wheat germ extracts were prepared according to Roberts and Paterson [20] except that potassium acetate was used in stead of potassium chloride. Wheat germ used for preparation of the extracts was kindly donated by Dr. van Kammen. Final extracts usually had an A_{260} of 95 units/ml with an A_{260}/A_{280} ratio of 1.70. Small drops were frozen in liquid nitrogen and stored at −70 °C. Protein synthesis assays were performed in reaction volumes of 12 µl at the following final concentrations: 3 µl $30000 \times g$ supernatant, 20 mM Hepes (pH 7.6 with KOH), 8 mM creatine phosphate and 40 µg ml creatine kinase (both obtained from Boehringer), 1 mM ATP, 80 µM GTP, 75 mM KOAc, 2.5 mM MgOAc, 2 mM dithioerythritol, 200 µM spermine, 5 mM of each of the amino acids except [^{35}S]methionine (300 Ci mmol, Radiochemical Centre, Amersham) of which 0.2 mCi ml was added. Spermine reported to give an additional stimulation in the wheat germ system [21] did have a positive effect on protein synthesis but analysis of the products did not show any specificity for the high-molecular-weight polypeptides. Mixtures were incubated at 25 °C for 60 min. Total incorporation was determined by adding trichloroacetic acid to 2 µl aliquots, heating the mixtures for 2 min at 80 °C and collecting the precipitate on Whatman GF/A filters. After drying filters were counted in a toluene-based scintillant.

Gel Electrophoresis

Aliquots of protein extracts from glands and tissue culture cells and the products of the protein synthesis *in vitro* were made 0.06 M in Tris-HCl (pH 6.8), 5%

in dodecylsulphate, 5% in 2-mercaptoethanol, 10% in glycerol and 0.001% in bromphenol blue in a final volume of 20 µl. These samples were then heated at 80 °C for 2 min and analyzed on 10% acrylamide/dodecylsulphate slab gels [7].

Proteins were also analyzed by two-dimensional gel electrophoresis [22]. Isoelectrofocussing in the first direction was performed in cylindrical gels [23]. Gels contained 5% acrylamide and 2% ampholine carriers pH 3.5–10 (LKB) in 8 M urea. As cathode and anode buffer, 0.02 M NaOH (degassed) and 0.01 M H_3PO_4 were used respectively. Gels were run for 18 h at 150 V. After they had been equilibrated in 0.0625 M Tris-HCl (pH 6.8), 5% 2-mercaptoethanol, 2.3% dodecylsulphate, 10% glycerol for 3 h at 23 °C, gels were layered on a 10% acrylamide/dodecylsulphate slab [7] and run in the second direction. After drying, gels were fluorographed [24] by exposure to Kodak RP/R 14 X-ray film.

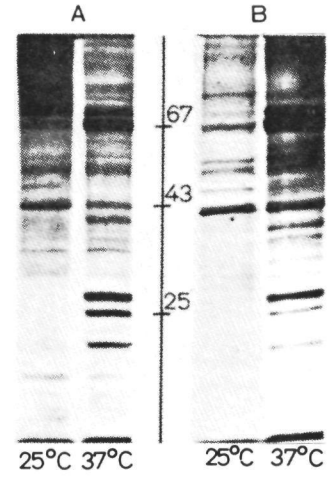

Fig. 1. *Autoradiograms of [^{35}S]methionine-labelled protein pattern of salivary glands of* D. hydei. (A) Glands were incubated at 25 °C for 15 min in incomplete Poels' [18] medium with 2 µCi/µl [^{35}S]methionine. In the case of heat shock, glands were incubated at 37 °C for 60 min followed by a 20-min labeling period at 25 °C. (B) Dechorionated 12-h-old embryos were incubated at 37 °C for 60 min, injected with 40 nCi [^{35}S]methionine and further incubated at 25 °C for 20 min. Markers ($M_r \times 10^{-3}$) are indicated in between the two lanes

RESULTS

Heat-Shock Peptides in vivo

Incubation at 37 °C of *D. hydei* tissue results in the appearance of a number of newly synthesized bands in the protein pattern as analyzed by dodecylsulphate/polyacrylamide gel electrophoresis. Heat-shocked salivary glands showed six bands that appear after a 30-min temperature treatment and continue to be synthesized for many hours (Fig. 1 A, see also: Fig. 6 and 7).

To investigate whether more than six polypeptides were induced, ^{35}S-labelled extracts of heat-shocked glands were analyzed by two-dimensional electrophoresis and the pattern obtained was compared with that of untreated glands (Fig. 2 and Table 1). The most prominent spot on the autoradiogram represents the 67000-M_r heat-shock protein. Close to it the 70000-M_r product is seen as a weak spot. The 38000-M_r product seen in Fig. 1 could no longer be detected in the two-dimensional pattern owing to the relatively low amount of label incorporated in this peptide. The lower-molecular-weight region contains three major spots representing the 26000-M_r, 25000-M_r and 20000-M_r heat-shock bands as seen on the usual dodecylsulphate gels. Also a number of minor spots appear on these patterns. However, their intensity is variable and they do not always migrate as clearly distinct spots, see, for instance, the two satellite products on both sides next to the major 26000-M_r and 20000-M_r proteins. Thus, the six heat-shock bands seen in dodecylsulphate gels of salivary glands contain a number of additional polypeptides, which differ in their isoelectric point or molecular weight from the major heat-shock proteins. These may represent distinct polypeptides or some of them may be derived from others by post-translational processing.

The protein-synthetic response to heat shock of tissues other than salivary glands has been shown to be qualitatively analogous to the response of the latter tissue [5, 14]. Indeed, the one-dimensional dodecylsulphate gel electrophoretic pattern of an ^{35}S-labelled extract from heat-shocked *D. hydei* embryonic cells is identical to that from salivary glands (compare Fig. 1 A and B). Two-dimensional analysis of the extracts reveals, however, that one more major protein (M_r about 26000, isoelectric point 7.7) is induced in heat-shocked embryonic cells (Fig. 3 A). The same product can also be found in extracts of total first-instar larvae and imaginal discs or ventral ganglia of third-instar larvae after heat-shock treatment. The dodecylsulphate gel electrophoretic pattern of heat-shocked tissue-culture cell extracts does differ from that of salivary glands (Fig. 4): one extra heat-shock band appears at 25500 M_r and in some cell lines the 20000-M_r protein is no longer present. The isoelectric point of the 25500-M_r protein in a two-dimensional pattern (Fig. 3 B) is identical to the isoelectric point of one of the proteins with a molecular weight of about 26000 seen in embryos and some other larval tissues but not in glands. This suggests that these proteins are identical and that relatively poorer resolution of fluorography, used to analyze the peptide patterns from embryos, has obscured the slight molecular

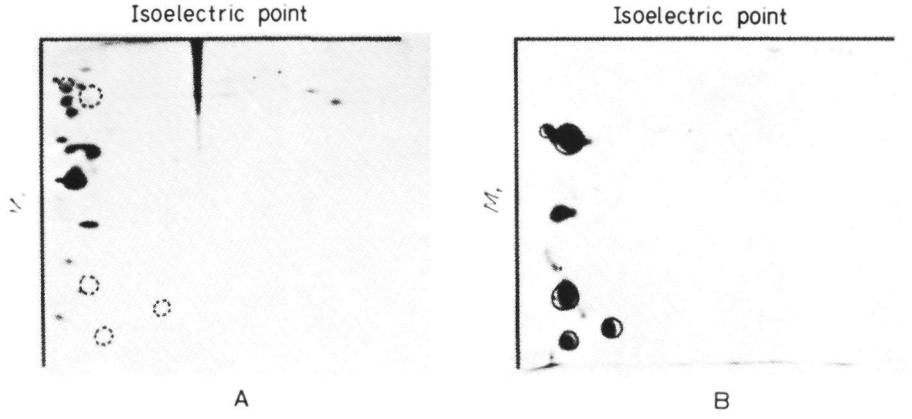

Fig. 2. *Autoradiographs of two-dimensional electropherograms of [^{35}S]methionine-labelled salivary glands incubated at 25 °C or 37 °C.* Labelling procedure was as described in the legend of Fig. 1. First direction (horizontal axis) consisted of isoelectric focussing in a pH gradient, followed by 10% polyacrylamide dodecylsulphate electrophoresis in the second direction. Results have been summarized in Table 1. Circles indicate the major heat-shock peptides while corresponding positions in the control pattern are marked with broken circles. Gels were fluorographed according to Bonner and Laskey [24]

Table 1. *Identification of heat-shock proteins in salivary glands, embryos and tissue-culture cells on two-dimensional gel electropherograms*
The table summarizes the results from experiments such as those shown in Fig. 2, 3 and 6. The position of the heat-shock proteins *in vivo* is determined from two-dimensional patterns. They correspond with the positions of the polypeptides synthesized under the direction of RNA isolated from embryos or tissue-culture cells. The minor heat-shock proteins have been indicated with brackets. Especially these proteins do not always appear as clearly separated spots; their isoelectric point and intensity depend on the tissue and varies slightly from one experiment to another. Data in the last column of this table indicate the size of RNAs coding for the heat-shock proteins

$10^{-3} \times M_r$	Isoelectric point	Presence *in vivo*	Synthesis *in vitro* and size of RNA
70	4.4	present in all tissues examined	directed by 20-S RNA from embryos and tissue-culture cells
67	5.0	present in all tissues examined	directed by 20-S RNA from embryos and tissue-culture cells
(38)		minor band, not detected in 2-D gels	probably 20-S RNA
(33)		minor band, seen only in tissue culture cells	probably 20-S RNA
26	4.8 (4.7, 4.9)	all tissues examined	13-S RNA from embryos and tissue-culture cells
25500	7.7 (6.2, 7.2, 7.9)	not found in salivary glands	13-S RNA from embryos and tissue-culture cells
25250	(5.8)	all tissues examined	13-S RNA from embryos and tissue-culture cells
25000	5.8 (5.6)	all tissues examined	13-S RNA from embryos and tissue-culture cells
20000	4.6 (4.5, 4.7)	not present in two cell lines	RNA from the corresponding lines does not code for this protein; 13-S RNA for embryos and other cell lines.

weight difference between the embryonic 26000-M_r and 25500-M_r peptides. The absence of the 20000-M_r protein is not tissue specific, since two out of our four independently isolated cell lines do induce this polypeptide after a heat shock (Fig. 4). Small differences also exist in the relative intensities and positions of the minor heat-shock products when the different cell lines are compared. Table 1 summarized the results obtained with glands, embryos and tissue-culture cells. It shows that different *D. hydei* tissues show a qualitatively very similar response of their protein synthetic pattern after a heat-shock treatment. The only relevant difference we have been able to find is a 25500-M_r protein in tissue-culture cells and embryos which is not induced in salivary glands.

This difference could be due to post-translational processing of one of the peptides or could be encoded by mRNA. These two alternatives may be distinguished if the mRNA is translated *in vitro*, since, if it is due to a tissue-specific post-translational processing, then one would expect to find only the precursor protein *in vitro*.

Protein Synthesis in vitro

To compare the protein synthetic patterns *in vivo* and *in vitro* of the heat-shock peptides, RNA was prepared from polysomes isolated from embryos or from tissue-culture cells. It is clear from the patterns *in vivo* that the heat-shock bands are the major protein products at 37 °C. This shift from the synthesis of many proteins at 25 °C to a few at 37 °C is reflected in the sedimentation profile of the polysomes of *D. hydei* cultured cells (Fig. 5). After transfer to 37 °C

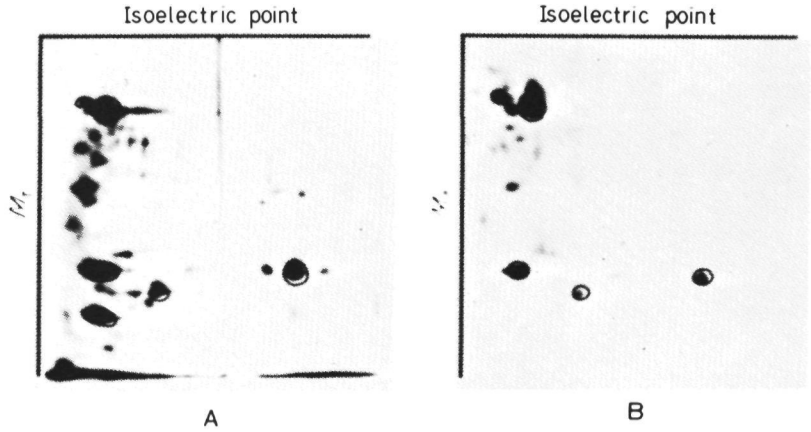

Fig. 3. *Autoradiographs of two-dimensional electropherograms of [^{35}S]methionine-labelled protein extracts of heat-shocked* D. hydei *embryos and tissue-culture cells.* Extracts were prepared as described in Materials and Methods. Circles indicate the major heat-shock peptides (see also Table 1). (A) The two-dimensional pattern of embryos; (B) the pattern of one of the cell lines which did not induce the 20000-M_r protein

a new profile is established in approximately 30 min, which is characterized by a high percentage of 80-S monosomes and a small shift of the remaining polysomes to higher sedimentation values (20–25 ribosomes/mRNA). This profile remains constant for at least the next 2 h. The drastic shift in size distribution of polysomal material, such as seen in *D. melanogaster* tissue-culture cells [14], does not occur in *D. hydei* cells. This is consistent with differences in the intensity of the heat-shock bands in *D. hydei* and *D. melanogaster* tissue-culture cells: in the latter more than half of the total incorporated [^{35}S]methionine label appears in the 70000-M_r band, while in *D. hydei* tissue-culture cells the 67000-M_r and 70000-M_r bands together account for only 25% of the total incorporated label.

Poly(A)-containing RNA was prepared from polysomes using poly(U)-Sepharose chromatography. For tissue culture cells 3.5% of the total polysomal RNA was bound to the poly(U)-Sepharose and could be eluted with 90% formamide at 65 C. This RNA was then used to direct protein synthesis in a wheat germ extract. Under optimal conditions about 40000 counts min^{-1} μl^{-1} were incorporated corresponding to a 20-fold stimulation above the endogenous level of protein synthesis. The total incorporation was the same whether RNA was isolated from heat-shocked or control cells and depended only on the concentration of the RNA added. To show any difference in coding properties of RNA isolated from treated and non-treated tissue, products of the wheat germ incubation were analyzed by dodecylsulphate gel electrophoresis.

The pattern encoded by RNA from heat-shocked cells *in vitro* clearly contains the heat-shock peptides: both high-M_r and low-M_r bands seen *in vivo* after a heat-shock treatment are also present in the pattern *in vitro*. These are not present in the pattern encoded by RNA isolated from control cells (Fig. 4). Two-dimensional analysis of the wheat germ products (Fig. 6A) shows that all heat-shock proteins synthesized under the direction of RNA isolated from treated tissue-culture cells are the same, with respect to molecular weight and isoelectric points, as those found *in vivo*. The pattern *in vitro* obtained with RNA from embryos in a wheat germ incubation (data not shown) also fits exactly the one observed *in vivo*, including the 25500-M_r protein with isoelectric point of 7.7 and the minor heat-shock proteins appearing in the low-molecular-weight region.

The relative synthesis of the two high-molecular-weight bands was much lower *in vitro* than *in vivo*. The latter pattern is a function not only of the rate of initiation of the respective mRNA species but also reflects the accumulation of these peptides during the labelling period. The pattern *in vitro*, on the other hand, should be determined only by the relative concentrations and initiation frequencies of the mRNA species involved. Of course, the efficiency of initiation on the different mRNAs may also differ between *Drosophila* cells and wheat germ extracts. Furthermore, the wheat germ system is known to be relatively inefficient in the translation of long mRNAs and can give rise to a series of smaller, discrete, peptides due to premature termination of the peptide chain [25]. To determine whether any of the shorter peptides observed in our system *in vitro* are due to a process of premature termination of longer mRNAs, poly(A)-containing RNA from heat-shocked tissue-culture cells was separated according to size on a sucrose

gradient and the various fractions were assayed in the wheat germ translation system *in vitro*. As shown in Fig. 7 and summarized in Table 1, two peaks of mRNA activity were observed, one around 20 S and one around 13 S. Analysis of the protein products of these RNAs showed that the 20-S region contained the coding sequences for the high-molecular-weight heat-shock peptides, probably including the 38000 and 33000-M_r proteins (Fig. 7). The size of the RNA coding for the smaller heat-shock peptides could not be determined unambiguously from this pattern, since the 20-S RNA fraction also directs the synthesis of polypeptides with molecular weights of about 25000. Therefore, products synthesized *in vitro* under the direction of 13-S heat-shock RNA were analyzed

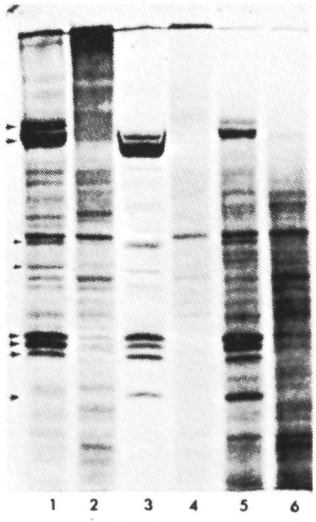

Fig. 4. *Protein pattern of* D. hydei *tissue-culture cells labelled* in vivo *compared with the pattern of proteins synthesized* in vitro *under the direction of poly(A)-containing RNA isolated from cells (see also Table 1)*. Extracts were prepared as described in Materials and Methods. Lanes 1 and 2 show the protein synthetic pattern of heat-shocked and control tissue-culture cells that do not induce the 20000-M_r protein. Lanes 3 and 4 show the corresponding patterns of another cell line where this protein is present. The last two lanes show the products synthesized in a translation assay *in vitro* under the direction of RNA isolated from heat-shocked and control cells of the type presented in lane 3. Heat-shock bands are indicated with small arrows beside the lanes

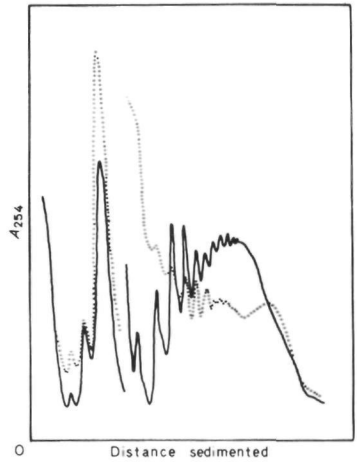

Fig. 5. *Effect of heat shock on the polysomal profile of* D. hydei *tissue-culture cells*. (——) Profile of cells grown at 25 °C. After cells have been shifted to 37 °C a new profile (·····) is established in about 30 min. No further changes occurred during an additional 2-h incubation at 37 °C. Absorbance scale is expanded 5-fold after the 80-S peak

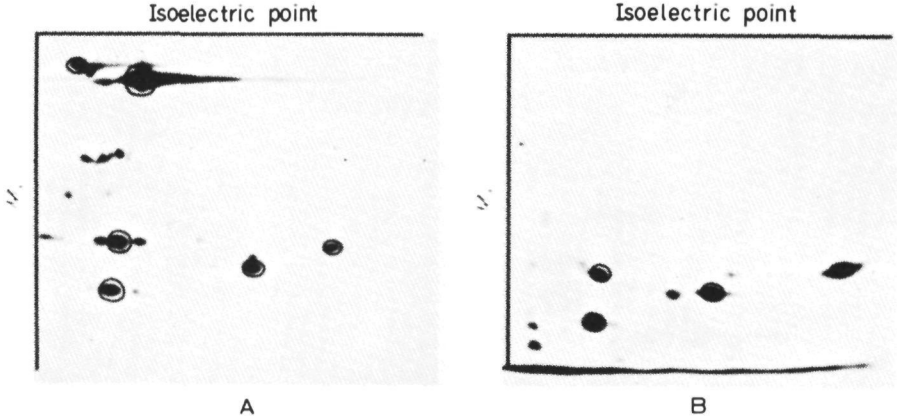

Fig. 6. *Autoradiographs of two-dimensional electropherograms of products synthesized* in vitro *in response to heat-shock RNA from tissue-culture cells*. Samples, such as those used in Fig. 4 lane 5, were analyzed by two-dimensional electrophoresis. Heat-shock peptides *in vitro* are indicated with circles. (A) The products synthesized under the direction of poly(A)-containing RNA from heat-shocked culture cells; (B) the pattern obtained if only the 13-S poly(A)-containing RNA fraction is added to the wheat germ incubation

by two-dimensional electrophoresis (Fig. 6B). This pattern contains all low-molecular-weight heat-shock proteins. These are thus encoded by RNA with a size of about 13 S. This does not exclude the possibility that some of the small products *in vitro*, especially those whose synthesis is stimulated by 20-S RNA (Fig. 7), are a result of a phenomenon such as premature termination. On the other hand, they may also represent wheat-germ-specific proteins, since the 20-S RNA from control cells also stimulates the synthesis of these polypeptides. Further studies, for example by peptide mapping, will be necessary to determine whether these products *in vitro* correspond to those found *in vivo* or whether they are artefacts of the translation system *in vitro*.

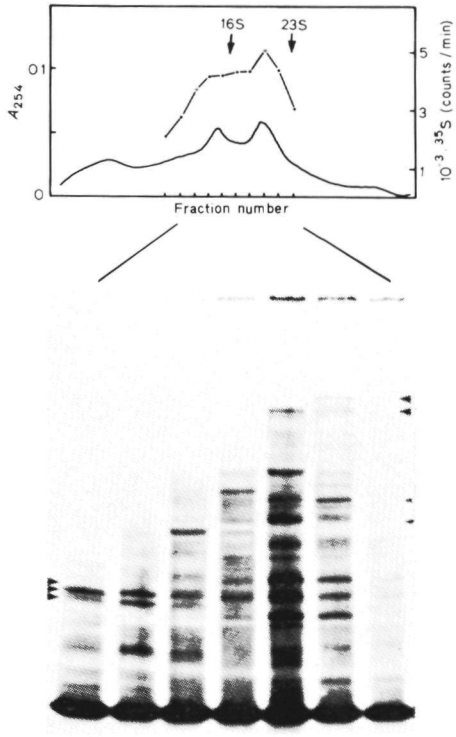

Fig. 7. *Sedimentation analysis of poly(A)-containing RNA from heat-shocked* D. hydei *tissue-culture cells.* Poly(A)-containing RNA was prepared from polysomes of heat-shocked tissue-culture cells and layered on linear sucrose dodecylsulphate gradients. Samples were centrifuged in an IEC SB-408 rotor for 4 h at 55000 rev. min. Relevant fractions were precipitated with alcohol and assayed in wheat germ extracts. The lower part shows the products synthesized under the direction of differently sized RNAs. Results have been summarized in Table 1. (——) Absorbance at 254 nm; (·—·—·) the total incorporation in response to the RNA fraction

DISCUSSION

Drosophila tissues that have been submitted to a temperature treatment show a number of drastic changes in their protein synthetic pattern *in vivo*: most of the proteins synthesized at 25 °C cease to be made, and six new bands account for most of the [^{35}S]methionine incorporation as analyzed by autoradiography of dodecylsulphate gels [5–7]. Two-dimensional electrophoresis of labelled cell extracts reveals that the situation is more complex: in salivary glands at least two of the bands give rise to multiple spots on autoradiographs. Comparison of the response of salivary glands and tissue-culture cells showed that most of the spots found in the autoradiographs of the electropherograms of heat-shocked tissue-culture cells coincide with those of salivary gland cells, but some peptides are found in one tissue only. For example, in tissue-culture cells a few peptides unique to the heat-shocked cells are found that do not appear in salivary glands. One of these (at 33000 M_r) is visible only on a one-dimensional electropherogram just as the 38000-M_r peptide in salivary glands. More obvious is the occasional lack of a 20000-M_r peptide and the presence of major and some minor 25500-M_r peptides in tissue-culture cells. The latter are also found as major and minor spots in embryonic patterns, in which tissue they do not separate clearly from the 26000-M_r peptide on dodecylsulphate gels. To determine whether any of these differences is due to possible tissue-specific post-translational modifications, the synthesis of these peptides in wheat germ extracts was analyzed. Poly(A)-containing RNA isolated from different cell lines, as well as embryos, directed the synthesis *in vitro* of all heat-shock proteins found *in vivo* in corresponding tissues. Molecular weights and isoelectric points determined from two-dimensional patterns were the same *in vivo* and *in vitro*. Also the low-molecular-weight minor heat-shock proteins, including the satellite products of the 26000-M_r, 25500-M_r and 20000-M_r major proteins were synthesized in the wheat germ system. The synthesis of the 25500-M_r polypeptide *in vitro*, stimulated by embryonic and culture cell RNA, makes it rather unlikely that it could arise from post-translational modification of, for example, the 26000-M_r polypeptide. Furthermore, gradient analysis has shown that there is a small difference in size of the RNA coding for the 26000-M_r and 25500-M_r proteins. The 25500-M_r major heat-shock product in culture cells must, therefore, be coded for by a separate RNA. Similarly the lack of synthesis of the 20000-M_r product in two cell lines must be due to an absence of the appropriate mRNA.

With the methodology used here, we cannot be certain whether the minor satellite heat-shock products are also encoded by separate RNAs or whether they

are derived from the major proteins by a post translational modification such as N terminal acetylation *in vivo* as well as *in vitro*. Further characterization by peptide mapping may solve this problem.

The presence of some of the polypeptides *in vitro* which do not belong to the heat shock products may be due to processes such as premature chain termination. The size of the presumptive mRNAs coding for these peptides is rather large (around 20 S) considering the molecular weights (from 70000 to 20000) and this finding together with the low synthesis of the high molecular weight peptides relative to the pattern *in vivo* would support the occurrence of premature termination.

From the translation and sedimentation analysis *in vitro* of the RNAs coding for the major heat shock peptides it is apparent that there must be at least six distinct mRNA species: the one for the 70000-M_r band sediments slightly ahead of the one for the 67000 M_r band, while the four mRNAs for the 26000, 25500, 25000 and 20000 M_r bands all sediment around 13 S. There should thus (again excluding the minor heat-shock bands) be at least six genes active in tissue culture cells.

A heat-shock activates four loci on *D. hydei* salivary gland chromosomes as indicated by the formation of puffs. Messenger RNA complementary to these same loci is also found in heat shocked tissue culture cells and, as we have argued elsewhere [16], the amount of DNA at these loci complementary to the tissue-culture RNA sequences should be sufficient to provide the information for the major heat shock proteins. However, as we have shown here, the heat shock pattern from the tissue culture cell line used in these studies lacks one of the salivary gland peptides and a new peptide is found instead *in vivo* as well as *in vitro*. Obviously then some of the genes activated in tissue culture cells are not identical to those of salivary gland cells. Either this peptide is not encoded for by a heat-shock locus, and indeed only in the case of the 70000-M_r peptide do we have any direct evidence that this peptide is a product of one of the heat shock loci (namely 2–36 A [28]), or alternatively the gene for this protein could be located closely linked to one of the heat shock sensitive sites. Furthermore, autoradiography of pulse labelled heat shocked salivary gland chromosomes has shown a high background activity over the whole genome [26], while after hybridization *in situ* sites other than the heat-shock loci are labelled ([27, 28] and unpublished observation) suggesting that a number of additional loci are transcribed. These sites may code for some of the minor proteins.

The activation of the heat shock sensitive loci may be a response of the cell to metabolic stress. As a result synthesis *de novo* of some enzymes involved in the mitochondrial metabolism is found, presumably in order to alleviate this stress (for review see [12]). Considering the variety of isoenzymes known to occur in *Drosophila* (for review see [29]), it would not be surprising if some of the heat shock peptides showed tissue-specific differences. We would suggest that this is the case for the appearance of a 25500 M_r protein in tissue culture cells and embryos *in vivo*. Furthermore, the same product is also found in heat shocked first instar larvae and in the imaginal discs and ganglia from third-instar larvae. On the other hand, the absence of the 20000 M_r protein is cell line specific: other *D. hydei* cell lines do synthesize this product *in vivo* and *in vitro*. It may be pointed out here that this phenomenon is not associated with eventual aneuploidy of some of the cell lines, since the karyotypes show no irregularities as far as concerning the autosomes.

In the foregoing discussion we have assumed that the mRNAs for the heat shock peptides do not preexist in the cell but are made exclusively at 37 C or at least, that the concentration of these mRNAs at 25 C is low enough not to affect the specific activity of the mRNA species isolated from heat shocked cells incubated with radioactive precursors. Only if this assumption is correct will hybridization of such RNA preparations *in situ* indicate the sites coding for the heat-shock peptides. It has been shown that treatment with actinomycin D prevents the appearance of the heat shock peptides [6, 7, 14] and the expression of the heat-shock peptides thus depends upon RNA synthesis *de novo*. On the other hand, it is clear from the synthetic patterns *in vivo* and from the polysome patterns ([14] and as shown above) that a heat shock is also accompanied by a translational response: only the heat shock mRNAs are actively translated. This is in marked contrast to the situation during incubation with arsenite, for example, in this case the peptides made in control cells continue to be synthesized [7], although arsenite activates the same loci and the heat shock peptides also appear. Spradling et al. [27] have shown that the amount of pre existing poly(A) containing RNA is strongly reduced during the heat shock treatment. Nevertheless, the heat-shock mRNA was not the predominant mRNA species. This is in agreement with our translation results *in vitro*: the heat shock peptides are a minor product. Apparently a number of mRNA species are still present in the polysomal preparations but are being poorly translated *in vivo*.

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CHAPTER VI

The Activity of Two Heat Shock Loci of *Drosophila hydei* in Tissue Culture Cells and Salivary Gland Cells as Analyzed by in situ Hybridization of Complementary DNA

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Abstract. Complementary DNA was made to poly A$^+$ nuclear or polysomal RNA isolated from heat shock tissue culture cells of *Drosophila hydei* A number of loci other than the four major heat shock loci are labelled after in situ hybridization of these cDNA preparations, while solution hybridization indicated that only about 10% of the cDNA was specific for heat shocked cells Removal of the fraction of cDNA which could react with 25° C RNA and subsequent in situ hybridization of heat shock specific cDNA indicated that locus 4-81 B, a major salivary gland heat shock locus, is also active at 25° C in tissue culture cells, while locus 4-85 B is specifically activated by heat shock in tissue culture cells This latter locus is not seen to be clearly puffed in salivary glands, but was shown to be active in that tissue both by direct autoradiography of salivary gland chromosomes after ^{3}H-uridine labeling and by hybridization of cDNA to chromosomal RNA

Introduction

Cytochemical studies of salivary gland polytene chromosomes have shown that interference with the respiratory metabolism of Drosophila cells (for example by incubation of the cells at 37° C instead of 25° C) not only causes the activation of the heat shock loci (Ritossa 1962 1964, Ashburner, 1970, van Breugel, 1966 Leenders and Berendes, 1972) but also the repression of the already active developmentally regulated loci (Jamrich et al , 1977) Thus, during incubation at 37° C the heat shock loci are the major sites of intense RNA synthesis while some RNA synthesis continues at other loci The changes in the pattern of transcription after a heat shock in diploid cells have been studied via in situ hybridization of in vivo labelled RNA (McKenzie et al 1975 Spradling et al , 1975, Bonner and Pardue, 1976 Lubsen et al , 1978) Since this technique detects only RNA species with high specific activity and of high concentration

the in situ hybridization pattern reflects only partially the transcription pattern of the cells. Nevertheless, it is clear that the major heat shock loci found in polytene chromosomes are also active in tissue culture cells during incubation at 37°C. However, a number of other loci, for example the histone locus (Spradling et al., 1975), are also seen to be active. Since we have shown that in *Drosophila hydei* the protein synthetic pattern during a heat shock differs between salivary glands and tissue culture cells (Sondermeijer and Lubsen, 1978), the question arises to what extent the transcription of loci other than the major salivary gland heat shock loci is unique to heat shocked cells. We have therefore prepared complementary DNA to both nuclear and polysomal poly A containing RNA from heat shocked cells, and reacted this cDNA with RNA from cells incubated at 25°C to remove those sequences which were also present before the temperature shift. The resulting cDNA preparations were then used in in situ hybridization experiments to identify those loci which are active only during a heat shock. These studies show that the major salivary gland heat shock locus, 4-81 B, is also active at 25°C in tissue culture cells and that locus 4-85 B, not known as a major salivary gland heat shock locus, is specifically activated by a heat shock in tissue culture cells.

Materials and Methods

The conditions for growth, labeling and fractionation of *D. hydei* tissue culture cells have been described previously (Lubsen et al., 1978; Lubsen and Sondermeijer, 1978). Poly-A+ polysomal RNA was prepared according to Sondermeijer and Lubsen (1978). To obtain poly-A+ nuclear RNA, RNA was isolated from the crude nuclear pellet by guanidine extraction as described by Strohman et al. (1977) and further purified by poly-U Sepharose chromatography as described for polysomal RNA.

Preparation and Hybridization of cDNA. ^{3}H-labelled cDNA was synthesized in the presence of ^{3}H-dCTP essentially as described by Monahan et al. (1976). The specific activity was 1.3×10^6 dpm/μg. Solution hybridization reactions in a final volume of 2 μl were carried out in 0.3 M NaCl, 20 mM Tris-HCl (pH 7), 1 mM EDTA, 0.1% SDS in sealed capillaries at 67°C. The extent of hybridization was assayed using S$_1$ nuclease according to Seeburg et al. (1977). cDNA was enriched for heat shock sequences by reaction with total 25°C cell RNA (final concentration 1.2 mg/ml) to a R_0t of about 100 in 50 μl final volume under the same conditions. Single stranded cDNA and cDNA-RNA hybrids were isolated from the large scale hybridization reaction by hydroxylapatite chromatography according to Kurtz and Engelson (1977).

In some experiments cDNA of defined size was used. This was obtained by centrifuging cDNA through a 15 to 30% sucrose gradient in 10 mM Tris pH 7.2, 0.1 M NaCl, 10 mM EDTA, 0.2% SDS for 3 h at 10°C in an IEC SB 405 rotor. The 9S fraction was collected and cDNA was recovered by alcohol precipitation in the presence of *E. coli* ribosomal RNA as carrier.

In situ Hybridization. Conditions for in situ hybridization and quantitation of the patterns obtained have been described previously (Lubsen and Sondermeijer, 1978). cDNA hybridization to chromosomal RNA was performed as described by Livak et al. (1978).

Materials. All radioactive materials were obtained from the Radiochemical Centre, Amersham. Reverse transcriptase was generously donated by Dr. J.W. Beard. S1 nuclease was from Sigma, hydroxylapatite grade HTP was from BioRad and *E. coli* ribosomal RNA was obtained from Miles. All other chemicals used were reagent grade.

Results

In situ Hybridization and Solution Hybridization of cDNA

Complementary cDNA prepared from either poly A^+ nuclear RNA ($cDNA_n$) or from poly A^+ polysomal RNA ($cDNA_p$) hybridized, as shown in Figure 1, to the known salivary gland heat shock loci of *Drosophila hydei*, namely 2-32A,

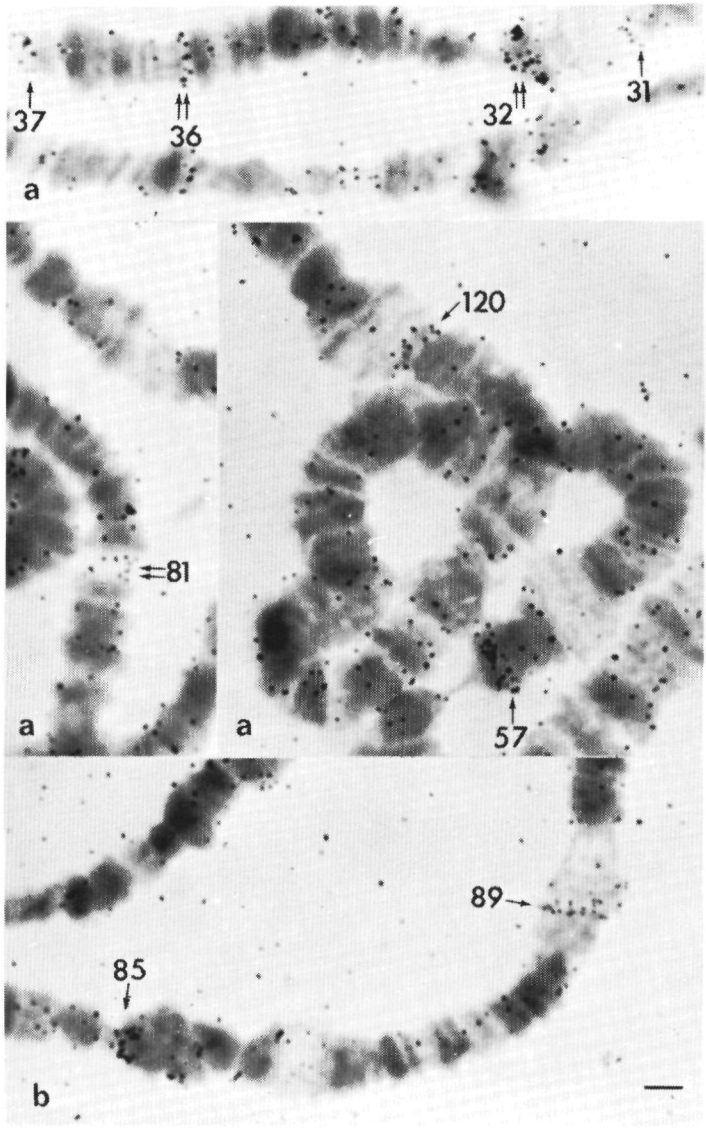

Fig. 1a and b. In situ hybridization of 9S cDNA. **a** $cDNA_p$ was hybridized at a concentration of 2.5×10^4 cpm/μl. Exposure time was 35 days. **b** $cDNA_p$ hybridized as in a, but exposure time was 46 days. Chromosomal loci are indicated by the number of the band. Major heat shock loci are shown with a double arrow

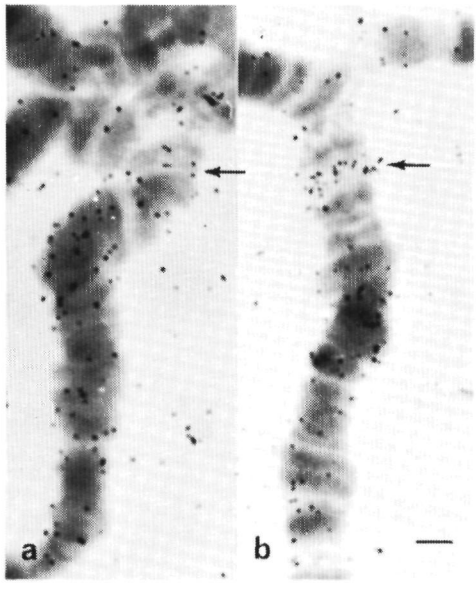

Fig. 2a and b. In situ hybridization of 9 S cDNA to locus 2-48C. **a** $cDNA_p$ was hybridized at a concentration of 2.5×10^4 cpm/μl. Exposure time was 35 days. **b** $cDNA_n$ was hybridized at 6.4×10^3 cpm/μl. Exposure time was 46 days. The arrow indicates locus 2-48C

2-36A, 2-48C and 4-81B (for review, see Berendes, 1975) and the relative grain densities over these loci are about the same as those found using in vivo labelled RNA (see Lubsen et al., 1978): high density over locus 2-32A, much lower over 2-36A and 4-81B, while locus 2-48C is strongly labelled using $cDNA_n$, but only weakly with $cDNA_p$ (Fig. 2). In vivo labelled heat shock RNA hybridizes, in addition to the four major heat shock loci, also markedly to loci 4-85B and 3-59A. cDNA only hybridized to one of these, namely 4-85B, but not to locus 3-59A. The RNA complementary to locus 3-59A does not contain poly-A (unpublished observation) thus explaining the lack of the corresponding cDNA. Furthermore, cDNA hybridizes to a number of other sites on the chromosomes (for example: 2–31, 2–37, 3–57, 4–89, 5–120, see Fig. 1) and a general label over the chromosomes is seen.

The cDNA sequences hybridizing to loci other than the heat shock loci could be complementary to RNA already present in the cell before transfer to 37°C, since the majority of polysomal RNA isolated from heat shocked cells does not code in vitro for the heat shock proteins, the major in vivo translation products (Sondermeijer and Lubsen, 1978).

To determine the extent of reaction of our cDNA preparations with 25°C RNA, cDNA was hybridized in solution with 25°C RNA and the kinetics of the reaction were followed. Both $cDNA_p$ (Fig. 3a) and $cDNA_n$ (Fig. 3c) reacted extensively with 25°C RNA, namely to about 80%. The extent of reaction of $cDNA_p$ and $cDNA_n$ with their template (Fig. 3b, d), heat shock poly-A^+ nuclear or polysomal RNA, was not significantly different from the extent of reaction of the cDNA with 25°C RNA, nor did the kinetics of the reaction differ significantly, if one takes into account that total 25°C RNA and not poly-A^+ RNA was used. These data do not show the presence of a heat shock

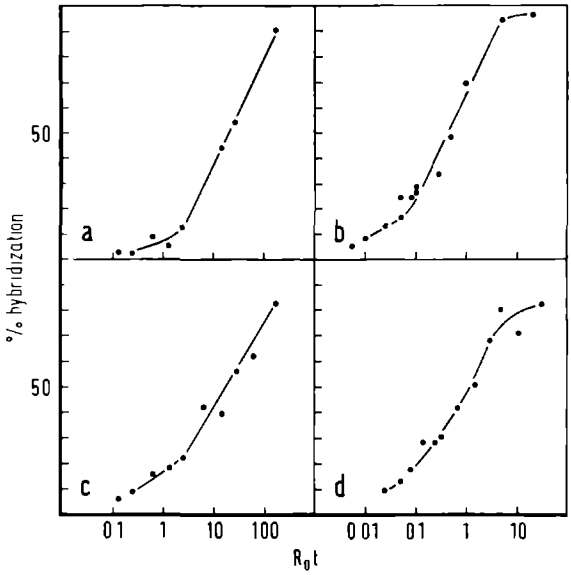

Fig. 3a–d Kinetics of hybridization of cDNA and RNA in solution **a** $cDNA_n$ with total 25°C RNA **b** $cDNA_t$ with poly A polysomal RNA **c** $cDNA_t$ with total 25°C RNA **d** $cDNA_n$ with poly A nuclear RNA

Table 1. Extent of reactions of fractionated cDNA with heat shock RNA cDNA preparations were incubated with total 25°C total polysomal or total nuclear heat shock RNA as indicated to R_0t of 150 The extent of reaction was assayed by hydroxylapatite chromatography

	% hybridized
cDNA with total 25° RNA	79
ss $cDNA_t$ with total polysomal RNA	30
cDNA which did not hybridize to total 25°C RNA and to total polysomal RNA with total nuclear RNA	35
$cDNA_t$ with total 25°C RNA	83
ss $cDNA_t$ with total polysomal RNA	50

specific cDNA fraction However, solution hybridization of the total cDNA preparations with the various RNA preparations may not be sensitive enough to detect a minor fraction Therefore to enrich for possible heat shock specific sequences, the cDNA preparations were reacted with 25°C RNA to R_0t of 150, unreacted cDNA (ss cDNA) was separated from the cDNA-25°C RNA hybrid by hydroxylapatite chromatography and then incubated with total heat shock polysomal RNA, again to R_0t 150 As shown in Table 1, both $cDNA_n$ and $cDNA_n$ reacted to about 80% with 25°C RNA Of the 20% of the $cDNA_n$ which did not react with 25°C RNA, 30% was capable of further reaction with total heat shock polysomal RNA About a third of the fraction of $cDNA_n$

Table 2. Quantitation of in situ hybridization of ss and ds $cDNA_n$. Grain counts and their standard deviation shown were obtained, after 18 days exposure, with ds $cDNA_n$ at 1.13×10^3 cpm/μl and ss $cDNA_n$ at 9.3×10^2 cpm/μl. In situ hybridization patterns at two other cDNA concentrations were also quantitated and the relative concentration of the various sequences was obtained from the ratio of the slopes of a double reciprocal plot of grain counts versus cDNA concentration

Locus	Grain counts ± s.d.		Relative concentration ss cDNA/ds cDNA
	ds cDNA	ss cDNA	
2-32A	39±21	78±29	1.7
2-36A	12±12	25±13	3.5
2-48C	20±14	35±15	1.8
4-81B	23±15	16±16	0.7
4-85B	15±16	32±22	1.8

which did not react with either 25°C RNA or heat shock polysomal RNA, could hybridize further with nuclear heat shock RNA. Thus, of our $cDNA_n$ preparation, 80% is also present in 25°C RNA, a further 6% is not present in 25°C RNA but is shared with polysomal heat shock RNA, while 5% is only found in heat shock nuclear RNA. Similarly, 83% of $cDNA_p$ is common to both 25°C RNA and heat shock polysomal RNA, and only 8% is unique to heat shock polysomal RNA.

A similar approach was used to identify the active loci unique to heat shocked cells by in situ hybridization of cDNA: cDNA was reacted with total RNA extracted from control cells to a R_0t of about 100. About 60% of the cDNA hybridized with RNA under these conditions and this cDNA (ds cDNA) was removed from the non reacting species (ss cDNA) by hydroxylapatite chromatography. Both these cDNA preparations were then used for in situ hybridization. Hybridization to the four known salivary gland heat shock loci and to locus 4-85B, the only other locus significantly labelled with ss cDNA, was quantitated. An example of the grain counts obtained using ds and ss $cDNA_n$ is shown in Table 2 (similar results were obtained using $cDNA_p$). Hybridization was quantitated at different cDNA concentrations, these results were plotted in a double reciprocal plot of grain counts vs cDNA concentration (Lubsen and Sondermeijer, 1978) and the slope of the resulting lines was used to calculate the relative concentration of a particular cDNA species in ds and ss cDNA. As shown in Table 2, ss cDNA was about twofold enriched for sequences complementary to loci 2-32A, 2-36A, 2-48C and 4-85B, but was relatively depleted for sequences complementary to heat shock locus 4-81B. These data show that a heat shock activates locus 4-85B in tissue culture cells, while locus 4-81B is apparently also active at 25°C. However, we do detect in vivo labelled heat shock RNA hybridization to this locus and this must thus also be active at 37°C. If unlabelled 4-81B sequences are indeed present before transfer of the cells to 37°C then the specific activity of in vivo labelled 4-81B RNA will be lower than that of for example 2-32A RNA which is not present in control cells. A comparison of the kinetics of RNA and cDNA (of which,

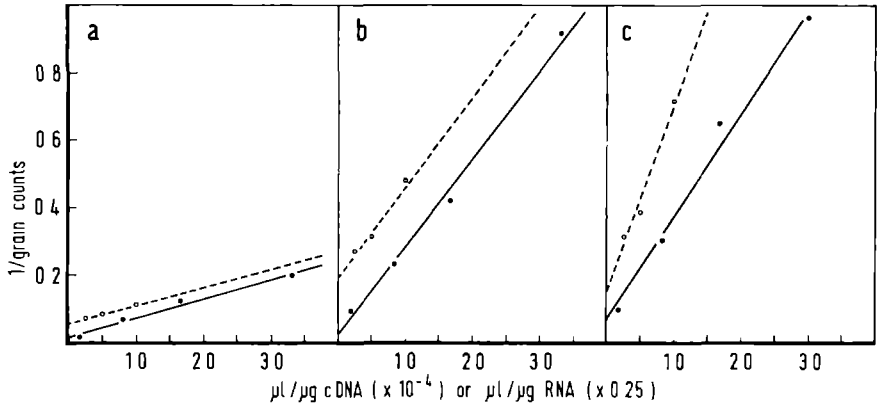

Fig. 4a–c. Quantitative in situ hybridization of heat shock RNA and $cDNA_p$. Double reciprocal plot of grain counts versus RNA or cDNA concentration. All grain counts were standarized to 40 d exposure. Ordinate scales were chosen such that for 2-36A (**b**) the RNA-line parallels the cDNA line. Corresponding lines for 2-32A and 4-81B are in **a** and **c** resp. Broken lines represent the polysomal RNA (s.a. 4×10^3 cpm/µg). Solid lines represent the $cDNA_p$ (see also Table 3)

Table 3. Relative concentration of heat shock sequences in cDNA compared to their concentration in RNA. Slopes were calculated from the data as shown in Figure 4. Last column of this table shows the apparent relative enrichment of 4-81B sequences in cDNA preparations

Locus	Slope of RNA-line (µg/µl × 10^3)	Slope of cDNA-line (µg/µl × 10^6)	Concentration of labelled sequences in cDNA relative to RNA (× 10^{-3})
32A	(13.2 ± 0.3)	(5.7 ± 0.3)	(2.3 ± 0.2)
36A	(70.5 ± 5.7)	(26.0 ± 1.2)	(2.7 ± 0.3)
81B	(138.3 ± 13.2)	(30.4 ± 1.7)	(4.5 ± 0.7)

of course, length and specific activity of all sequences are the same) hybridization allows the calculation of the relative specific activity of different RNA species and should show a relatively lower specific activity of 4-81B RNA. Accordingly, ^{3}H-labelled polysomal poly-A$^+$ RNA was isolated and part of this preparation was used for the synthesis of cDNA and part was hybridized in situ directly. Both RNA and cDNA preparations were hybridized in situ at different concentrations, grains were counted over the various loci and plotted against RNA or cDNA concentration in a double reciprocal plot (Fig. 4). Since the $C_0t_{1/2}$ and the $R_0t_{1/2}$ should remain constant, assuming no preferential copying by reverse transcriptase, both the slope and the intercept of these lines are a function of the specific activity of the RNA. In our calculation we have used the slope, since this value is less sensitive to error (Lubsen and Sondermeijer, 1978). In Figure 4, where the results of such an experiment are shown, the scale was chosen such that the slopes of the RNA and cDNA lines are the same for locus 2-36A. Then the slopes of the RNA and cDNA lines for locus 2-32A are also parallel indicating that 2-32A and 2-36A RNA have the same specific

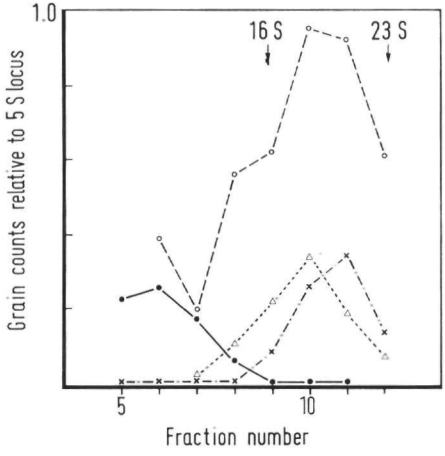

Fig. 5. In situ hybridization of different sized RNA fractions. Poly-(A)-containing RNA (s.a. 1×10^5 cpm/μg) from polysomes of heat-shock tissue culture cells was separated according to size on sucrose/dodecylsulphate gradients as described for cDNA in Methods. ^{3}H-5S RNA (s.a. 9×10^5 cpm/μg) was added and after alcohol precipitation fractions were hybridized in situ. Grain counts of the heat shock loci 2-32A, 2-36A, 4-81B and 4-85B were standarized to the saturated 5S locus 2-23B. Size markers were 16 and 23 S ribosomal RNA from *E. coli*

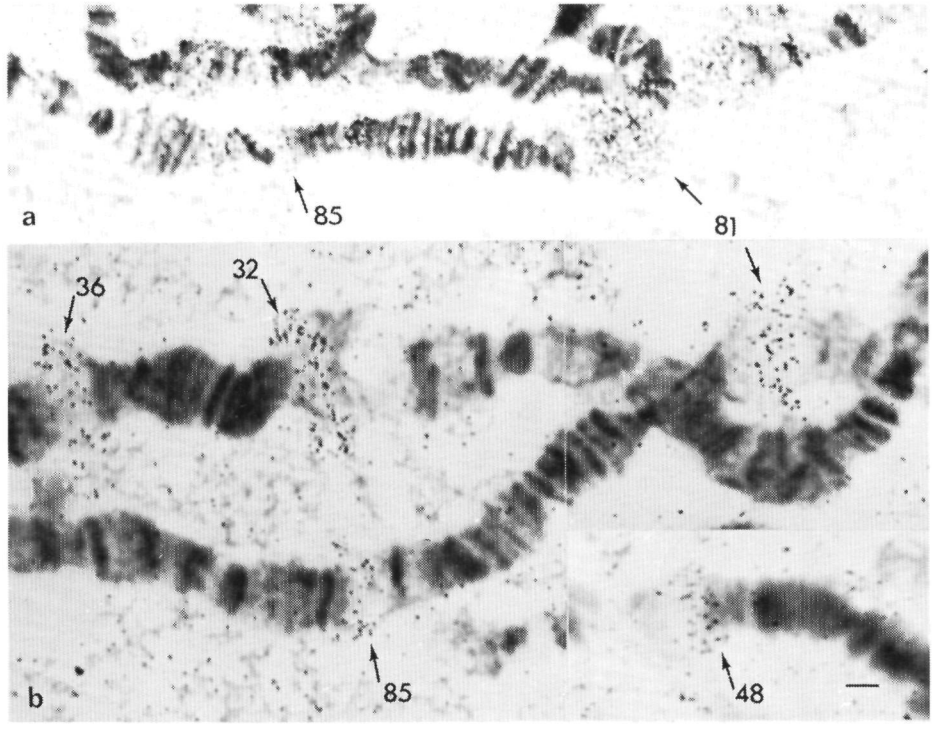

Fig. 6. a Transcription autoradiograph of D. hydei salivary gland polytene chromosomes. Salivary glands were isolated from late third instar larvae, incubated for 1 h at 37° C in the medium described by Shields and Sang (1977) which we also use for our tissue culture. Subsequently glands were incubated for 10 min in medium containing 100 μCi/ml ^{3}H-uridine and prepared for autoradiography as described by Berendes (1968). Exposure time was three days. **b** In situ hybridization of $cDNA_n$ to chromosomal RNA. $cDNA_n$ at 1.8×10^4 cpm/μl was hybridized to chromosomes from heat shocked salivary glands under conditions favoring DNA-RNA hybridization (see Methods). Exposure time was 17 days. Heat shock loci are indicated

activity. The slope of the RNA line for locus 4-81B is twice as large as that of the cDNA line and the specific activity of 4-81B RNA is thus half of that of 2-32A and 2-36A RNA and this RNA must thus be also present in control cells, in agreement with our ss cDNA hybridization data. This calculation is valid only if 2-32A, 2-36A and 4-81B RNA are all of the same length. To show that this is indeed the case, ^{3}H-labelled poly-A$^+$ polysomal RNA was separated by sucrose gradient centrifugation and fractions of the gradient were used for in situ hybridization As shown in Figure 5, 2-36A and 4-81B are both approximately 20S, the main peak of RNA hybridizing to locus 2-32A is also 20S but a minor, 13S, component is present. Locus 4-85B and 2-48BC both produce smaller RNAs.

The Activity of Locus 4-85B During Heat Shock in Salivary Glands

The data obtained with in situ hybridization of ss cDNA show that locus 4-85B is activated by heat shock in tissue culture cells This locus has been previously reported as a heat shock locus in salivary glands, but only during a specific stage of development (Berendes et al., 1966), and this locus has therefore not been considered a major heat shock locus in D. hydei. In the light of our results with tissue culture cells, we have reexamined the activation of this locus by a heat shock in salivary glands Autoradiography of heat shocked salivary glands pulse labelled with ^{3}H-uridine shows that this locus is indeed active in all salivary glands of all stages examined although never clearly puffed (Fig 6a). When cDNA prepared from tissue culture cell RNA is used as a probe for chromosomal RNA bound to salivary gland chromosomes, this locus is also labelled, as are the heat shock puffs (Fig 6b), showing that indeed the same RNA is produced in both tissue culture cells and salivary glands

Discussion

We have previously shown that, in *D. hydei*, the synthesis of one of the heat shock proteins is tissue specific, since we were unable to detect the synthesis of the 25,500 dalton protein found in tissue culture cells in salivary glands (Sondermeijer and Lubsen, 1978). Hence, it is not surprising that the RNA synthesis pattern at 37°C also shows tissue specificity. This is indicated by the high activity of locus 4-85B in tissue culture cells: according to our cDNA in situ hybridization data the amount of RNA complementary to this locus present in tissue culture cells is about the same as that complementary to locus 2-36A, while, if puff size is taken as a measure of activity in salivary glands, this locus is much less active than locus 2-36A there. Locus 4-85B produces a 13S RNA, the same size as that of the RNA coding for the 25,500 dalton heat shock protein (Sondermeijer and Lubsen, 1978), and the apparent difference in activity of this locus in these two tissues could thus be related to the differential synthesis of this protein At the present time, however, we have no direct evidence that locus 4-85B does code for the 25,500 dalton protein.

In contrast to locus 4-85B locus 4-81B is also active at 25°C in tissue culture cells while this locus is, at least when studied cytologically inactive at 25°C in salivary glands Since we do not know the half life of 4-81B RNA in tissue culture cells, we cannot determine the extent of activation of this locus in heat shocked cells However, we have observed that, in cells labelled for one hour the specific activity of 4-81B RNA is about half that of the other heat shock RNA, thus the amount synthesized in one hours is approximately equal to the amount present in the cell before transfer to 37°C If the half life of this RNA would be the same as that found for short lived mRNA species in *D melanogaster* cells, namely one hour (Lengyel and Penman, 1977), then this locus would not be activated by a heat shock but merely not repressed

In some experiments, such as the one shown in Figures 2 4, we have used cDNA of defined size for in situ hybridization The amount of grains over any one locus at saturation is then a measure of the number of genes present at that locus From the intercept of the double reciprocal plot of grain counts versus cDNA concentration, and using the hybridization efficiency determined by Szabo et al (1977), we estimate that locus 2 32A contains ten genes, locus 2-36A four, and that locus 2-48BC has only one gene coding for the RNA found in the cytoplasm Locus 2-48BC is always much heavier labelled using $cDNA_n$ than $cDNA_p$, and our preliminary data indicate that this denser label is due to the presence of sequences complementary to the nuclear 2-48BC RNA (Lubsen et al 1978), which does contain poly-A (unpublished observations) These data suggest that the poly-A containing sequences of the nuclear and cytoplasmic 2-48BC species are not identical, and the number of grains obtained indicate, furthermore that the nuclear 2-48BC RNA is repetitive either internally or externally

Our data on the kinetics of solution hybridization of cDNA with its template indicate a considerably higher complexity of heat shock polysomal RNA than that found by Biessmann et al (1978) using *D melanogaster* We have no real explanation for this discrepancy, and we can only suggest that, on the basis of published protein synthetic patterns of heat shocked *D melanogaster* tissue culture cells (McKenzie et al 1975, McKenzie and Meselson, 1977) and *D hydei* tissue culture cells (Sondermeijer and Lubsen, 1978), the translation control in the latter cells appears to be less stringent and the synthesis of all heat shock proteins about equal, while in the *D melanogaster* cells the main product appears to be the 70,000 dalton heat shock protein These effects would cause a higher complexity of *D hydei* polysomal RNA Clearly, a further investigation of the translational control and the fate of the preexisting mRNA in heat shock cells is needed to resolve this issue

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CHAPTER VII

Rapid Sequence Divergence in a Heat Shock Locus of *Drosophila*

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Abstract. In situ hybridization of cRNA transcribed from cloned *D. melanogaster* heat shock sequences to *D. hydei* chromosomes has shown that the *D. hydei* locus 2-32-A corresponds to the *D. melanogaster* locus 87 A/C and the *D. hydei* locus 2-36-A to the *D. melanogaster* locus 95 D, while the *D. hydei* locus 4-81-B corresponds to the *D. melanogaster* locus 63 BC. No hybridization to *D. hydei* chromosomes was found with cRNA transcribed from a clone containing the $\alpha\beta$ sequences encoded by the *D. melanogaster* locus 87 C. Neither *D. melanogaster* heat shock RNA nor *D. virilis* heat shock RNA hybridized significantly to the *D. hydei* heat shock locus 2-48-B. Furthermore, *D. hydei* heat shock RNA did not hybridize to the cytological homologs of locus 2-48-B found in *D. repleta* or in *D. virilis*. *D. hydei* heat shock RNA did hybridize to the cytological homologs of locus 2-48-B in *D. neohydei* and *D. eohydei*, both of which belong to the *hydei* subgroup.

Introduction

All *Drosophila* species studied so far have heat shock genes, i.e., a set of genes that is specifically induced by transferring tissue from 25° C to 37° C (for review, see Ashburner and Bonner, 1979). The characteristics of the heat shock response is similar in the various species studied more closely and it is generally believed that the heat shock genes in these species are homologous genes, i.e., that they are phylogenetically related. Indeed, it can be easily shown with the use of cloned DNA that the some heat shock genes of *D. melanogaster* and *D. hydei* are closely related (see Results). Some differences between species do however exist. For example, one of the heat shock loci in *D. hydei*, locus 2-48-B, can be induced with vitamin B_6 and produces a large and complex RNP particle (Leenders et al., 1973). Furthermore, it produces RNA that is mainly confined to the nucleus (Lubsen et al., 1978). Neither induction of a heat shock locus with vitamin B_6 nor production of a complex RNP particle by a heat shock locus have been found in *D. melanogaster* (Derksen, 1975), so that, no obvious cytological homology exists between locus 2-48-B of *D. hydei* and any of the heat shock sites in *D. melanogaster*. From linkage data

it could be surmised that locus 2 48 B of *D hydei* and heat shock locus 93 D of *D melanogaster* are related, since both map close to the *ebony* locus (Scalenghe and Ritossa, 1977, Lubsen, unpubl.) Alternatively, heat shock locus 87 C of *D melanogaster* could contain sequences homologous to locus 2 48 B since locus 87 C also produces RNA (the $\alpha\beta$ sequences) that is found only in the nucleus (Henikoff and Meselson, 1977) although others have detected it also in the cytoplasm (Lis et al. 1978) A second transcript of locus 87 C is virtually identical to the mRNA transcript of locus 87 A, both coding for a 70 000 dalton protein (Ish-Horowicz et al., 1979) Surprisingly, the $\alpha\beta$ sequences of locus 87 C are not found in *D simulans* which is closely related to *D melanogaster* although the 87 A/C mRNA sequences are present (Livak et al. 1978) It is thus of interest to determine whether the $\alpha\beta$ sequences are present in the much more distantly related *D hydei* and furthermore, to test whether heat shock RNA isolated from *D melanogaster* cells hybridizes to locus 2 48 B of *D hydei*

In contrast to the situation in *D melanogaster* induction of a heat shock locus with vitamin B_6 and production of the complex RNP particles is found in *D virilis* its locus 20 CD is cytologically homologous to locus 2 48 B of *D hydei* (Derksen, 1975 Gubenko and Baricheva 1979) Moreover within the *repleta* group to which *D hydei* belongs heat shock loci cytologically homologous to locus 2 48 B have been identified on the basis of the banding pattern of the polytene chromosomes (Berendes, 1965) By in situ hybridization of *D hydei* heat shock RNA to the polytene chromosomes of *D neohydei D eohydei D repleta* and *D virilis* and by in situ hybridization of *D virilis* heat shock RNA to *D hydei* we have tested whether the homology in cytology as found in the *repleta* group and in *D virilis* is accompanied by sequence homology of the RNA transcripts of these loci As we show below locus 2 48 B shows sequence homology only within the *D hydei* subgroup not elsewhere

Materials and Methods

^{3}H labeled RNA was isolated from heat shock tissue culture cells of *D hydei* and *D melanogaster* or from primary culture cells of *D neohydei* and *D virilis* as previously described (Lubsen et al. 1978) cRNA from plasmids 232.1 and 232.2 was kindly donated by M. Meselson cRNA from the other plasmids used in this study were prepared as described by Livak et al. (1978)

Conditions of in situ hybridization were as described previously (Lubsen et al. 1978) except that 6×SSC was used and the hybridization temperature was 60° C. The sites of hybridization of the polytene chromosomes were determined by using the following chromosome maps: for *D hydei* the map of Berendes (1965) for *D repleta* the map of Wasserman (1954) for *D virilis* the map of Hsu as published by Gubenko and Baricheva (1979)

Results

The Homology between Heat Shock Loci of D melanogaster and D hydei

DNA sequences of several of the *D melanogaster* heat shock loci have been cloned (for a description of the plasmids used here see Livak et al., 1978

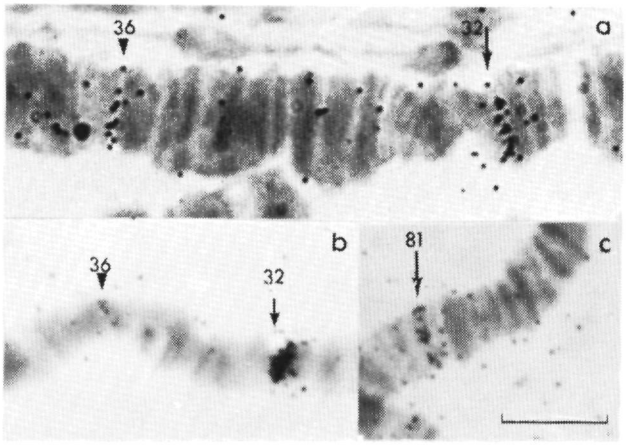

Fig. 1 a–c.[1] Hybridization of cloned *D. melanogaster* heat shock sequences to *D. hydei* chromosomes. **a** cRNA (1.1×10^5 cpm/μl) from plasmid pPW227 (*D. melanogaster* 95 D DNA), exposure time was 35 days; **b** cRNA (3.0×10^4 cpm/μl) from subclone 232.1 (*D. melanogaster* 87 C DNA), exposure time was 60 days; **c** cRNA (3.0×10^4 cpm/μl) from subclone 244.1 (*D. melanogaster* 63 BC DNA), exposure time was 60 days. In **a** and **b** the long arrow points to locus 2–32 A while the short arrow indicates locus 2–36 A. In **c** the long arrow points to locus 4–81 B

and Holmgren et al., 1979) and the presence of these sequences in the *D. hydei* chromosomes can be determined unambiguously by in situ hybridization. Accordingly, cRNA from three different clones of *D. melanogaster* heat shock loci were hybridized to *D. hydei* salivary gland chromosomes: cRNA from subclone 232.1, which contains the coding sequence for the 70,000 dalton heat shock protein and which derives from the *D. melanogaster* locus 87 C, hybridized readily to locus 2–32 A and to a much lesser extent to locus 2–36 A (Fig. 1 b). The latter locus hybridized well with cRNA from clone pPW 227, which derives from the *D. melanogaster* locus 95 D. Hybridization to locus 2–32 A was also seen with this cRNA (Fig. 1 a). These data show that the *D. melanogaster* loci 87 A/C are homologous to the *D. hydei* locus 2–32 A while the *D. melanogaster* locus 95 D corresponds to the *D. hydei* locus 2–36 A. Furthermore, there is sequence homology between loci 2–32 A and 2–36 A as is found for loci 87 A/C and 95 D in *D. melanogaster* (Holmgren et al., 1979). Finally, the *D. hydei* locus 4–81 B hybridized with cRNA from subclone 244.1, which contains the coding sequence from *D. melanogaster* locus 63 BC (Fig. 1 c). In contrast, cRNA from subclone 232.2 (which contains the $\alpha\beta$ sequence encoded by the *D. melanogaster* locus 87 C) did not hybridize at all to any site of the *D. hydei* chromosome. This sequence thus appears to be absent in *D. hydei*.

Since no cloned DNA from locus 2–48 B of *D. hydei* is available, we could not directly determine the presence or absence of this sequence in *D. melanogaster* DNA. We have therefore determined whether such a sequence can be found in *D. melanogaster* heat shock RNA. ^{3}H-labeled nuclear RNA was extracted from heat shocked *D. melanogaster* tissue culture cells and hybridized to *D. hydei* chromosomes. Grains were counted over locus 2–32 A, which served as

[1] Bars in all figures represent 10 μm.

Table 1.

a Hybridization of *D. melanogaster* heat shock RNA to *D. hydei* chromosomes. *D. melanogaster* nuclear heat shock RNA (specific activity 6.7×10^4 cpm/μg) or *D. hydei* nuclear heat shock RNA (specific activity: 3.1×10^4 cpm/μg) was hybridized to *D. hydei* chromosomes as described in Methods. Grain counts (±standard deviation) shown in the table were corrected to 37 days exposure. *n* indicates the number of nuclei counted

RNA	Conc (cpm/μl)	Grain counts		n
		Locus 2-32 A	Locus 2-48 B	
D. melanogaster	2.1×10^4	8.3±2.1	0.7±0.7	12
	1.0×10^4	5.5±1.6	0.6±0.5	14
D. hydei	7.5×10^4	22.1±3.0	12.7±1.2	9
	9.4×10^3	14.0±4.8	7.5±3.3	12

b Competition between labeled *D. hydei* RNA and cold nuclear *D. melanogaster* RNA. *D. hydei* heat shock nuclear RNA was hybridized at the concentration shown in the Table in the absence or presence of added *D. melanogaster* cold nuclear heat shock RNA to *D. hydei* chromosomes as described in Methods. The specific activity of the *D. hydei* RNA was 3.7×10^4 cpm/μg, exposure time was 18 days. Grain counts shown (±standard deviation) are the average of 10 nuclei

μg *D. hydei* ^{3}H RNA	μg *D. melanogaster* RNA	Grain counts		n
		Locus 2-32 A	Locus 2-48 B	
2.8	–	13.0±3.0	12.8±2.3	10
2.8	8.0	7.6±1.4	12.2±3.2	10

a hybridization control, and over locus 2-48 B. As expected, locus 2-32 A showed significant labeling with *D. melanogaster* RNA. However, little label was seen over locus 2-48 B (Table 1a). Similar results were obtained using polysomal RNA (data not shown). When a three fold excess of unlabeled *D. melanogaster* heat shock nuclear RNA was added to labeled *D. hydei* nuclear RNA, grain counts over locus 2-32 A were reduced by about 50% while no significant reduction of grain counts over locus 2-48 B was observed (Table 1b). These data cannot rule out the presence of a sequence homologous to the nuclear 2-48 B sequence in the *D. melanogaster* genome, but if such a sequence is present, it must be transcribed at a much lower rate than the other heat shock loci, while in *D. hydei* this transcript is found in a relatively high concentration in the nucleus of heat shocked cells (Lubsen et al., 1978).

Cross Hybridization of Heat Shock RNA between D. hydei, D. neohydei, D. eohydei, and D. repleta

D. neohydei belongs to the same subgroup as *D. hydei*. The salivary gland chromosome maps are identical (Berendes, 1965). When nuclear heat shock RNA from *D. hydei* was hybridized to salivary gland chromosomes from *D.*

Table 2.

a Cross hybridization between *D. hydei* and *D. neohydei* heat shock loci *D. hydei* nuclear RNA (s.a. 1.4×10^5 cpm/μg) was hybridized at 1.3×10^5 cpm/μg, while *D. neohydei* nuclear RNA (s.a. 2.0×10^4 cpm/μl) was hybridized at 6.6×10^4 cpm/μl. Exposure time was 31 days. Grain counts shown are the average (± standard deviation) of 20 nuclei

Source of RNA	Grain counts						
	D. hydei			*D. neohydei*			n
	2-32 A	2-48 B	32 A/48 B	2-32 A	2848 B	32 A/48 B	
D. hydei	28.8 ± 5.3	18.2 ± 3.2	1.6	20.2 ± 3.0	11.1 ± 1.6	1.8	20
D. neohydei	27.3 ± 5.6	21.5 ± 2.8	1.3	14.5 ± 2.9	14.4 ± 2.8	1.0	20

b Cross hybridization between *D. hydei*, *D. eohydei* and *D. repleta* heat shock loci *D. hydei* nuclear heat shock RNA (s.a. 7×10^5 cpm/μg) was hybridized to *D. hydei*, *D. eohydei*, or *D. repleta* chromosomes at 3.8×10^4 cpm/μl. Exposure time was 79 days. Grain counts shown are the average (± standard deviation) of the number of nuclei counted shown under n

Chromosomes	Grain counts			
	2-32 A	2-48 B	32 A/48 B	n
D. hydei	29.4 ± 7.1	28.7 ± 6.1	1.0	14
D. eohydei	20.0 ± 6.3	13.2 ± 5.8	1.5	20
D. repleta	23.1 ± 7.4	0.8 ± 1.0	28.9	11

neohydei, the hybridization pattern obtained was identical with that obtained when *D. hydei* chromosomes were used (Table 2a). Similarly, when nuclear RNA extracted from heat shocked *D. neohydei* primary cultures was hybridized to either *D. hydei* or *D. neohydei* chromosomes, the same hybridization patterns were obtained. Clearly, there is extensive homology between the heat shock loci of *D. hydei* and *D. neohydei*. *D. eohydei* also belongs to the *hydei* subgroup but is somewhat more distantly related to *D. hydei* than *D. neohydei*: Interspecies hybrids can still be obtained, but in a very low yield (Hennig, 1978). The polytene chromosome maps are still identical (Berendes, 1965). When *D. hydei* nuclear RNA was hybridized to *D. eohydei* chromosomes, locus 2–32 A was relatively more strongly labeled than locus 2-48 B, while the same RNA preparation gave an approximately equal labeling of these loci on *D. hydei* chromosomes (Fig. 2, Table 2b). These data indicate that either the sequence at the 2-48 B loci have diverged more than the 2-32 A sequences, or that there are more gene copies at the 2-32 A locus of *D. eohydei* than in *D. hydei*. At present we cannot distinguish between these two alternatives. In any case, some divergence in heat shock sequences has occurred.

D. hydei, *D. neohydei*, and *D. eohydei* all belong to the *hydei* subgroup of the *repleta* group, while *D. repleta* belongs to the *melanopalpa* subgroup of the *repleta* group and is more distantly related. Nevertheless, the polytene chromosome maps can be homologized and the heat shock puffs are located

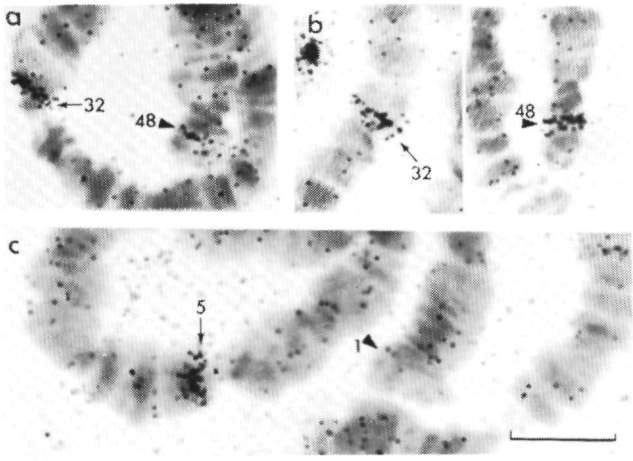

Fig. 2a–c. Hybridization of nuclear heat shock RNA from *D. hydei* to *D. hydei*, *D. eohydei* and *D. repleta* chromosomes. **a** *D. eohydei*; **b** *D. hydei*; **c** *D. repleta*. Locus 2–32 A or 2–5 D (*long arrow*) and locus 2–48 B or 2–A 1 (*short arrow*) are indicated. For the conditions of hybridization, see legend to Table 2b

at the same sites (Berendes, 1965). When the sequence homology of these loci is tested by hybridizing nuclear *D. hydei* heat shock RNA to *D. repleta* chromosomes, locus 2–D 5, homologous to locus 2–32 A, shows strong hybridization, while locus 2–A 1, homologous to locus 2–48 B, shows no hybridization at all (Fig. 2, Table 2b). Thus, although these loci are cytologically homologous, they contain different DNA sequences.

Cross Hybridization of Heat Shock RNA between D. hydei and D. virilis

D. hydei and *D. virilis* are too distantly related to allow one to homologize the various heat shock puffs merely on the basis of the chromosomal banding pattern. To analyze the sequence homologies of the heat shock transcripts, we started therefore by hybridizing nuclear and cytoplasmic RNA extracted from heat shocked *D. virilis* primary culture cells to *D. hydei* chromosomes. We quantitated the grain counts over locus 2–32 A and 2–48 B. As seen in Table 3 (and illustrated in Fig. 3b), significant hybridization was seen over locus 2–32 A but few grains were present at locus 2–48 B. When these RNA preparations were hybridized to the *D. virilis* chromosomes, three sites of strong hybridization were seen, all on the second chromosome: locus 29 C, locus 23 H, and locus 20 CD. Locus 29 C and 20 CD are heat shock loci (Poluektova et al., 1978; Evgen'ev et al., 1978; Gubenko and Baricheva, 1979). The hybridization to these two sites is shown in Fig. 3a and quantitated in Table 3. By in situ hybridization of cRNA from subclone 232.1 we confirmed the suggestion of Evgen'ev et al. (1978) that the *D. virilis* locus 29 C is the equivalent of the *D. melanogaster* loci 87 A/C and thus also of the *D. hydei* locus 2–32 A (Fig. 4a).

Table 3. Cross hybridization between *D. hydei* and *D. virilis* heat shock loci. *D. hydei* nuclear RNA was hybridized at 6.3×10^4 cpm/μl, the specific activity was 1.4×10^5 cpm/μg, while *D. virilis* nuclear RNA had a specific activity of 1.0×10^4 cpm/μg and was hybridized at 1.2×10^5 cpm/μl. *D. virilis* cytoplasmic RNA (s.a. 1.0×10^4 cpm/μg) was hybridized at 9.0×10^4 cpm/μl. Grain counts (±standard deviation) were corrected to 40 days exposure time. n indicates the number of nuclei counted

Chromosomes	RNA	Grain counts							
		D. hydei				*D. virilis*			
		32 A	48 B	32 A/48 B	n	29 C	20 CD	29 C/20 CD	n
D. hydei	nuclear	29.9±6.0	13.3±6.2	2.3	10	18.4±3.8	2.3±1.3	7.8	20
D. virilis	nuclear	17.1±3.9	3.4±2.0	5.0	10	17.6±4.9	23.3±4.9	0.8	10
D. virilis	cytoplasmic	12.8±2.9	1.7±1.0	7.4	20	18.0±4.3	24.2±2.7	0.7	10

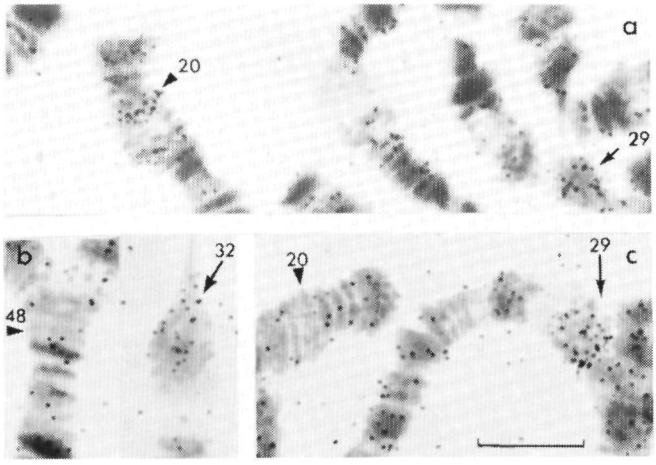

Fig. 3a–c. Cross hybridization of heat shock RNA between *D. hydei* and *D. virilis*. **a** *D. virilis* nuclear heat shock RNA to *D. virilis* chromosomes; **b** *D. virilis* nuclear heat shock RNA to *D. hydei* chromosomes; **c** *D. hydei* nuclear heat shock RNA to *D. virilis* chromosomes. Conditions of hybridization are described in the legend to Table 3, the long arrow indicates the loci 2–32 A or 29 C, the short arrow points to locus 2–48 B or 20 CD

Locus 23 H was identified as the histon locus by hybridization with cRNA of λh22, which contains the sea urchin histon genes (Schaffner et al., 1976) (Fig. 4b). The strong hybridization of *D. virilis* heat shock RNA to the third site, locus 20 CD, is reminiscent of the properties of the 2–48 B transcript, and this locus is indeed the heat shock puff of *D. virilis* that is, on cytological grounds, most likely to be homologous to locus 2–48 B of *D. hydei* (see Discussion).

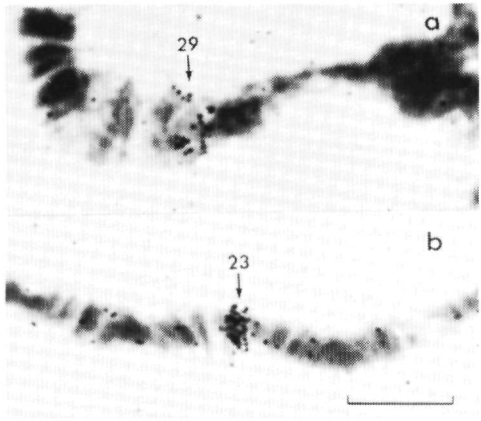

Fig. 4a and b. Localisation of cRNA from subclone 232.1 (*D. melanogaster* 87 C DNA) and λh22 (histon DNA) on *D. virilis* chromosomes. **a** cRNA from 232.1 at 1.2×10^4 cpm/μl. Exposure time was 36 days. **b** cRNA from λh22 at 1.2×10^5 cpm/μl. Exposure time was 36 days

We have also hybridized nuclear heat shock RNA from *D. hydei* to *D. virilis* chromosomes. As we have shown previously (Lubsen et al., 1978), nuclear heat shock RNA shows strong hybridization only at three sites of the *D. hydei* chromosomes: 2–32 A, 2–48 B and 3–59 A (the histon locus). Since the homologs of two of these loci in *D. virilis* are known, this RNA preparation can be used to probe for the presence of a third site of strong hybridization of the *D. virilis* chromosomes, which would be indicative of a homolog of locus 2–48 B. However, no such hybridization at a third site was detected, hybridization was found only on loci 29 C and 23 H. Although the putative homolog of the *D. hydei* locus 2–48 B, locus 20 CD, showed little hybridization, we quantitated the grain counts over that site and compared these with the number of grains over locus 29 C (Table 3). Only the hybridization to the latter locus was significant, not that to locus 20 CD. Thus, if locus 2-48 B and locus 20 CD are indeed homologs, as they appear to be from their cytology, then they do not contain similar DNA sequences.

Discussion

Since *D. melanogaster* does not contain a heat shock locus that is cytologically similar to locus 2–48 B of *D. hydei,* it is perhaps not surprising that little hybridization of *D. melanogaster* heat shock RNA to this site is seen.

Sequence correspondence would be expected to exist between the cytologically very similar loci 2–48 B of *D. hydei, D. neohydei* and *D. eohydei* and locus 2–A1 of *D. repleta* and 20 CD of *D. virilis*. This, however, is not found. We find complete correspondence of the 2–48 B heat shock loci only when *D. hydei* and *D. neohydei* are compared. Heat shock RNA from *D. hydei* already shows less hybridization to locus 2–48 B of *D. eohydei,* while no hybridization is found to the putative homologs of 2–48 B in *D. repleta* and *D. virilis*. This latter conclusion rests partially on the assignment of gene homology on the basis of corresponding banding patterns of the polytene chromosomes. This

procedure is jutified for the members of one subgroup (such as *D. hydei*, *D. neohydei*, and *D. eohydei*), but becomes more hazardous when members of one group are compared: Berendes (1965) has shown that the heat shock puff located at locus 2-36 A in *D. hydei* is probably translocated to locus 2-26 B in *D. mercatorum*, but no corresponding change in banding pattern could be seen at that site. In the same study, Berendes compared *D. hydei* and *D. repleta* and concluded that in this case the heat shock loci were located cytologically in the same chromosomal regions. The assumption that the *D. hydei* locus 2-48 B corresponds to the *D. virilis* locus 20 CD is based upon several facts: both contain complex RNP particles (Derksen, 1975; Swift, 1959), both are inducible by vitamin B_6 (Leenders et al., 1973: Derksen, 1975; Gubenko and Baricheva, 1979) and both are closely linked to the *ebony* locus (Evgen'ev et al., 1978; Lubsen, unpubl.).

It might be argued that the locus 2 48 B sequence does exist in *D. virilis*, but that it is not located at the expected site, namely 20 CD. Since we find little hybridization of *D. virilis* heat shock RNA to locus 2–48 B of *D. hydei*, this possibility is unlikely, unless the sequence is not transcribed during a heat shock. A similar argument can be made against the presence of the *D. virilis* 20 CD sequence in *D. hydei*: If it is present, it is not transcribed, otherwise hybridization of *D. hydei* heat shock RNA to locus 20 CD would be seen.

Thus, there seem to be two classes of heat shock RNA: one class codes for the major heat shock proteins and shows close sequence homology between various species. For example, the protein coding sequence of the *D. melanogaster* loci 87 A/C is found also in members of the *repleta* group and in *D. virilis*. Similarly, the coding sequences of the *D. melanogaster* loci 95 D and 63 BC are easily detected in *D. hydei*. The second class of heat shock transcripts shows little sequence homology, even between closely related species. Examples of this class are the $\alpha\beta$ sequences of locus 87 C of *D. melanogaster*, the nuclear transcript of locus 2 48 B of *D. hydei* and presumably the RNA produced by locus 20 CD of *D. virilis*. They do have in common that they do not appear to function as mRNA: A protein product of the $\alpha\beta$ sequences of locus 87 C could not be detected either genetically (Ish-Horowicz et al., 1979) or biochemically (Livak et al., 1978), while the nuclear location of the 2 48 B RNA precludes a mRNA function. The role of these RNA species in the heat shock response is unknown.

Besides the nuclear RNA, a second, probably mRNA, transcript from locus 2-48 B of *D. hydei* has been detected (Lubsen et al., 1978; Sondermeijer and Lubsen, 1979). This transcript contributes at most 20% of the grain density found over locus 2-48 B after in situ hybridization of nuclear heat shock RNA (Lubsen et al., 1978) and would therefore have gone undetected in the present study. It is quite possible that the various loci which show no homology when the nuclear 2 48 B sequence is considered, all contain this mRNA sequence and would show homology if the mRNA sequence could be studied.

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RNA products from locus 2-48B

Gene activity in Drosophila may be experimentally induced, for instance by increasing the temperature from 25° to 37°C.Such a heat shock treatment interferes drastically with the normal metabolism and causes a specific response at different levels in the cell (see introduction). It was our purpose to investigate those heat shock induced processes which occur at the level of RNA and protein synthesis. During the course of the work presented here we became primarily interested in the behavior of one specific site in the genome of D. hydei, namely locus 2-48B. The activity of this locus after heat shock can be visualized as a puff on the polytene chromosome of larval salivary glands. In this puff complex RNP particles are found, which are best seen after induction with vitamin B6. A 40S RNA species, which can be isolated from this puff by micromanipulation, may be a component of the complex RNP particle. As yet, nor the RNP particle, nor the 40S RNA have been biochemically purified since salivary glands are not a very suitable tissue to isolate RNA or RNP from due to the presence of mucopolysaccharides.
However, the heat shock response is shown by all tissues, including the cultured cell lines. This specific complex RNP particle can thus also be found in the nucleus of heat-shocked diploid cells. Analysis of the heat shock induced RNA transcripts complementary to 2-48B (chapter III) shows that in cultured cells some of these sequences are found only in the nucleus since in in situ hybridization, polysomal RNA could compete with only 20% of the nuclear RNA. Both RNA species are polyadenylated and the cDNAs copied from these RNAs probably differ in sequence (chapter VI). These data are consistent with the synthesis of a 40S RNA at locus 2-48B, although we have as yet not been able to detect such a large RNA in the nucleus of cultured cells. Hybridization experiments with cDNA (chapter VI) suggest that the organization of the nuclear RNA is complex. If one assumes a unique 40S transcript containing one poly-A tail at the 3' end, then a consistent difference in hybridization intensity should have been found when one compares in vivo

labelled nuclear and polysomal RNA with their corresponding cDNAs, since the 9S cDNA contains only 10% or less of the sequence of this presumptive nuclear RNA. However, no such difference is seen and thus the nuclear RNA sequence must be repetitive unless cDNA synthesis is primed internally due to secondary structures in the RNA molecule. Another alternative is that the 40S transcript is rapidly processed to smaller RNAs which are subsequently polyadenylated. This processing may well be blocked in the puff after vitamin B6 induction, thus yielding the 40S RNA which is found in 2-48B puffs isolated from salivary gland nuclei. Whether the 40S transcript is a multi-repeat of a single short sequence or whether it is composed of several non-identical sequences cannot be decided from the results presented in this thesis. Recently, all heat shock genes have been cloned via cDNA prepared from polysomal as well as nuclear heat shock RNA but, unfortunately, no data were available at the time this thesis was written.

The function of the nuclear 2-48B transcripts is unknown, and any hypothesis is limited by the fact that the sequences are relative unstable from a evolutionary point of view (chapter VII). The induced synthesis of nuclear transcripts is not just some peculiar property of locus 2-48B in D. hydei. Apart from the closely related species D. neohydei and D. eohydei, in which a more or less identical 2-48B RNA sequence can be detected, we have also found large transcripts at the homologous heat shock site in D. virilis. However, the sequence of this locus must have diverged largely or even been replaced by other ones since no cross hybridization with 2-48B in D. hydei is observed. Also in D. melanogaster, non protein coding sequences are transcribed from the heat shock loci. For instance locus 87C contains, apart from the sequence coding for the major heat shock polypeptide, the so-called $\alpha\beta$-elements, which belong to a family of multi-dispersed repetitive elements. These sequences are transcribed during heat shock and have been suggested to have a regulatory function in the synthesis of the heat shock proteins. Recently it has been reported that even the presumptive homolog of 2-48B in D. melanogaster, locus 93D, produces complex RNA transcripts which remain in the nucleus, although it has already been shown (chapter

VII) that there exists no sequence correspondence between these two loci.
Besides the nuclear transcript an additional RNA can be detected (chapter III) which hybridizes to 2-48B and probably functions as a messenger, coding for a minor heat shock protein since the concentration of this RNA is relatively low compared to those transcribed from 2-32A or 2-36A. From the data presented in chapter VII it cannot be concluded whether the gene coding for this cytoplasmic 2-48B RNA is conserved in evolution although one would expect it to be as stable as other protein coding heat shock sequences.
In summary, the heat shock induced RNA transcripts from locus 2-48B are shown to be a complicated phenomenon from a structural, a functional and an evolutionary point of view. Further elucidation of these problems could be achieved when cloned fragments of these sequences are available.

Heat shock response and the effect on protein synthesis

In chapter V the results are presented of the in vitro translation of heat shock RNA: the synthesis of all major heat shock polypeptides was stimulated in vitro by RNA species present in the polysomes of heat shocked embryos and cultured cells.
Some tissue specific differences were found which mainly concern the small polypeptides with a molecular weight of 20,000 and 25,500 dalton. Two-dimensional analysis of the heat shock proteins also revealed the presence of minor products some of which migrated close to a major polypeptide. These were found in vivo as well as in vitro. Still, this does not exclude the possibility that a heat shock protein is partially modified after translation, thereby producing its satellites. Recently we tested the occurrence of in vivo phosphorylation and acetylation, however, in both cases the results did not show such a post-translational modification of the heat shock proteins. Therefore it seems most likely that separate RNA species code for these closely related polypeptides which implies that the genes from which these RNAs are transcribed must be polymorphic. It may be mentioned here, that minor differences in the RNA sequence, even

a single base substition, can be sufficient to explain the shift in migration of these satellites on two-dimensional patterns.
Not only do a number of newly synthesized messengers appear but they are also translated preferentially during heat shock. Especially in salivary glands, it has been shown that the heat shock proteins are the major products synthesized during heat shock. However, from the data presented in chapter V it should be concluded that this effect in cultured cells is not as prominent as in glands. For instance, the cell-free translation of heat shock and control RNA shows that many of the species present at 25°C still can be found in the polysomes at 37°C, probably being poorly translated in vivo. Also the hybridization with cDNA, of which the data are presented in chapter VII, strongly indicate that the heat shock sequences will account for less than 10% of the total mRNA population in the polysomes at 37°C. This is in marked contrast with the situation in D. melanogaster in which a more stringent translational response is observed not only in salivary glands but also in some of the cultured cell lines.

Proteins coded for by the heat shock loci

The results presented in chapter V and VI suggest a correlation between specific heat shock loci and the products they code for. The 20S RNA fraction, containing sequences homologous to locus 2-32A, 2-36A and 4-81B, directs the synthesis of the 70,000 and 67,000 dalton polypeptides. The 13S RNA fraction, coding for the low molecular weight bands, shows hybridization to locus 4-85B and to some extent to locus 2-32A.
Unambiguously, these data and additional results on hybrid-arrested translation with cloned fragments of the heat shock loci have shown that the temperature induced sites, seen as puffs on the polytene chromosomes, each produce messenger RNA which codes for one of the heat shock proteins. The only exception to this rule is locus 2-48B of which the majority of the RNA transcript remains in the nucleus. In chapter IV we have found a correlation between locus 2-36A and the 70,000 molecular weight protein. However, our conclusions may have been somewhat premature since the interpretation of the data

assumed that the 67,000 molecular weight band contained only one polypeptide. Recent data from our laboratory have demonstrated that this band is composed of at least five polypeptides with small differences in isoelectric point. Furthermore, peptide mapping has shown that three of these have an almost identical amino acid sequence, while the other two resemble the first ones only in some part of their sequence. Therefore, it should also be possible to discriminate between the two sets of RNA species coding for these proteins. Such a situation has indeed been found in D. melanogaster for locus 87A/C and 95D, which showed about 15% divergence in their coding regions. Cloned fragments of these two loci in D. melanogaster also hybridize in situ to the respective heat shock sites in D. hydei, namely locus 2-32A and 2-36A. However, both also showed consistent cross hybridization as is demonstrated in chapter VII. The sequence homology between RNAs transcribed from these loci does not extent throughout the 3' end of the message, since plasmids constructed via the cDNA procedure do not show any cross-hybridization between 2-32A and 2-36A. Although the final evidence will have to be obtained from hybrid-arrested translation, it seems most likely that locus 2-32A and 2-36A will each produce a set of related messengers which together will code for all 67,000 molecular weight proteins.
Our previous suggestion in chapter IV, that 2-36A would code for the 70,000 molecular weight protein, therefore was not correct and in fact it has recently been shown that this polypeptide is encoded by locus 4-81B. This locus was found to be active also in control tissue culture cells (although perhaps not in control salivary glands , chapter VI) which means that the protein it codes for is present in control cells and may have an essential function in metabolism or development. Therefore, the concentration of this 70,000 molecular weight polypeptide in the original fraction used for raising the antibodies (chapter IV) must have been high enough to be antigenic which explains the specific reaction of the antiserum with the 70,000 molecular weight polypeptide (fig. 2, chapter IV). One would expect therefore to also have seen an increase in grain counts over locus 4-81B in this experiment. However, the grain density over this locus is low and was thus not analyzed.
In summary, it can be concluded from the data presented in this

thesis that the heat shock induced genes produce messengers which code for the heat shock proteins, with the exception of locus 2-48B which primarily codes for RNA that remains in the nucleus. Cloned fragments of these particular nuclear transcripts will elucidate their structure and perhaps also their function in the heat shock response.

RNA producten van locus 2-48B

In Drosophila kunnen genen experimenteel geinduceerd worden door bijvoorbeeld de temperatuur te verhogen van 25° C naar 37°C.Een dergelijke heat shock behandeling verstoort het normale metabolism in ernstige mate en leidt tot een specifieke respons op een aantal niveau's binnen de cel. Het doel van het hier beschreven onderzoek was na te gaan welke heat shock geinduceerde processen plaatsvinden met name op het niveau van RNA en eiwit synthese. Naarmate het werk vorderde werd de aandacht steeds meer gericht op specifiek één plaats van het D. hydei genoom, namelijk locus 2-48B. De activiteit van dit locus na een heat shock behandeling is microscopisch zichtbaar als een puff in de polytene chromosomen van larvale speekselkliercellen. Met name na inductie met vitamine B6 worden complexe RNP partikels in deze puff gevonden. Bovendien kan in door micromanupulatie geisoleerde puffs een 40S RNA aangetoond worden dat mogelijk een onderdeel vormt van dit RNP partikel. Geen van beiden is tot op heden biochemisch gezuiverd omdat uit speekselklieren om praktische redenen moeilijk RNA of RNP geisoleerd kan worden.
Andere weefsels, inclusief de permanente cellijnen, vertonen in principe dezelfde respons tijdens een heat shock behandeling. Vandaar dat het voor locus 2-48B specifieke RNP partikel ook in geinduceerde diploide cellen gevonden wordt. Analyse van de tijdens heat shock afgelezen RNA's in weefselkweekcellen, toonde aan (hoofdstuk III) dat een gedeelte van de aan locus 2-48B complementaire sequenties de kern niet verlaat. Beide RNA's worden gepolyadenyleerd en waarschijnlijk zijn er verschillen in de sequenties van de copie DNA's van kern en polysomaal RNA (hoofdstuk VI). Op zich zijn deze gegevens in overeenstemming met de synthese van een 40S RNA in puff 2-48B, alhoewel we tot nog toe geen RNA van een dergelijke grootte in de kern van weefselkweekcellen hebben kunnen vinden.
De resultaten van de cDNA hybridisaties doen vermoeden dat het kern RNA een complexe organisatie bezit. Indien we veronderstellen dat er een uniek 40S RNA afgelezen wordt met slechts één poly-A staart

aan het 3' einde dan zou een duidelijk verschil in de mate van hybridisatie gevonden moeten worden wanneer in vivo gemerkt kern en polysomaal RNA met de overeenkomstige cDNA's vergeleken worden omdat het 9S cDNA in dit geval slechts 10% van de sequentie van het vooronderstelde kern RNA bevat.
Aangezien een dergelijk verschil niet gevonden wordt moet het RNA repetitief zijn vooropgesteld dat de synthese van cDNA niet intern geinitieerd wordt als gevolg van secondaire structuren in het RNA molecuul. Een mogelijk alternatief is dat het 40S transcript snel verwerkt wordt tot een aantal kleinere RNA's die vervolgens gepolyadenyleerd worden. Deze processing zou geremd worden indien de inductie plaats vindt met 0.05 M vitamine B6 waardoor het 40S RNA zich in de puff kan ophopen. Aan de hand van de hier beschreven resultaten kan niet uitgemaakt worden of het grote kerntranscript is opgebouwd uit zichzelf repeterende korte sequenties dan wel uit meerdere niet-identieke sequenties. Dit is overigens een van de redenen geweest om de heat shock genen via hun cDNA's te cloneren. Helaas waren destijds de resultaten hiervan nog niet voldoende uitgewerkt om nog in dit proefschrift besproken te kunnen worden.

De funktie van het 2-48B kerntranscript is op dit moment nog onbekend. Het enige wat in dit verband opgemerkt zou kunnen worden, is dat deze sequenties evolutionair gezien niet stabiel zijn (hoofdstuk VII). Overigens is de synthese van een kerntranscript zeker niet specifiek voor locus 2-48B in D. hydei. Afgezien van de nauwverwante soorten D. neohydei en D. eohydei, waar min of meer identieke 2-48B RNA sequenties gevonden worden, bleek ook het homologe locus in D. virilis een groot kerntranscript te produceren. De sequentie van dit locus moet echter in hoge mate gedivergeerd zijn of mogelijk door een ander vervangen omdat geen kruishybridisatie met 2-48B in D. hydei kan worden waargenomen. Ook in D. melanogaster worden niet voor eiwit coderende sequenties van de heat shock genen afgelezen. Locus 87C b.v., bevat naast de sequentie voor het belangrijkste heat shock polypeptide ook nog de z.g. $\alpha\beta$-elementen. Deze verspreid in het genoom voorkomende gerepeteerde

sequenties worden eveneens gedurende de heat shock afgelezen en er zijn suggesties gedaan als zouden zij een regulerende functie hebben in de synthese van de heat shock eiwitten. Onlangs nog is gevonden dat ook het waarschijnlijk homologe locus voor 2-48B in D. melanogaster, locus 93D codeert voor complexe RNA's die de kern niet verlaten, waarbij opgemerkt dient te worden dat er, zoals aangetoond is in hoofdstuk VII, geen sequentie homologieen bestaan tussen deze twee loci.
Afgezien van het kerntranscript werd nog een tweede RNA gevonden (hoofdstuk III) dat hybridiseerde op 2-48B en waarschijnlijk een van de boodschapper RNA's is die coderen voor de minder prominente heat shock eiwitten omdat de concentratie van dit RNA relatief laag is vergeleken met die afkomstig van 2-32A of 2-36A. Aan de hand van de resultaten in hoofdstuk VII kan niet uitgemaakt worden of dit cytoplasmatische 2-48B RNA in de evolutie geconserveerd is gebleven alhoewel de verwachting is dat het net zo stabiel is als de andere eiwitcoderende heat shock sequenties.
Samengevat, volgt hieruit dat de heat shock geinduceerde RNA's van locus 2-48B qua structuur, functie en evolutionair gedrag een gecompliceerd fenomeen vormen. Vandaar dat gecloneerde fragmenten van deze sequenties in feite onmisbaar zijn om deze problemen op te kunnen lossen.

Het effect van heat shock op de eiwitsynthese

In hoofdstuk V zijn de resultaten beschreven van de in vitro translatie van heat shock RNA: de synthese van de belangrijkste heat shock polypeptides werd gestimuleerd door RNA's geisoleerd uit de polysomen van heat shock behandelde embryos of weefselkweekcellen.
Er werden enkele weefselspecifieke verschillen gevonden met name betreffende de polypeptides met een molecuul gewicht van 20.000 en 25.000 dalton. Twee-dimensionale analyse van de heat shock produkten liet zien dat sommige van de eiwitten op dit soort patronen opsplitsen in een aantal meer of minder geconcentreerde polypeptides die zeer nauwverwant aan elkaar zijn. Alhoewel dit verschijnsel met in vivo incubaties en met in vitro translaties gevonden werd, kan niet uitgesloten worden dat een heat shock protein na vertaling gemodifi-

ceerd zou worden om aldus een of meerdere satellieten op te leveren. Experimenten waarbij met name naar in vivo phosphorylering en acetylering werd gekeken hebben onlangs uitgewezen dat de heat shock eiwitten in dat opzicht niet gemodificeerd worden na vertaling. Het meest waarschijnlijke is daarom dat er verschillende RNA's zijn die elk coderen voor een van de nauwverwante polypeptides hetgeen tevens betekent dat de genen waar deze RNA's van worden afgelezen polymorfie zouden moeten vertonen. In dit verband kan nog opgemerkt worden dat minimale sequentie verschillen, zelfs substitutie van een enkele base, voldoende kunnen zijn om het migratieverschil van satelliet produkten op twee dimensionale patronen te verklaren.
Een heat shock induceert de synthese van een aantal nieuwe boodschapper RNA's en bovendien worden deze RNA's preferentieel vertaald gedurende de behandeling. Met name in speekselklieren zijn de heat shock eiwitten de belangrijkste produkten die aangemaakt worden. De resultaten beschreven in hoofdstuk V wijzen echter uit dat dit effect in weefselkweekcellen minder is dan in speekselklieren. De in vitro translatie van heat shock en controle RNA laat zien dat een groot gedeelte van de boodschappers aanwezig bij 25°C terug gevonden wordt in de polysomen bij 37°C, alhoewel zij dan waarschijnlijk minder efficient vertaald worden. Ook de resultaten verkregen met cDNA hybridisaties (hoofdstuk VII) geven aan dat de heat shock sequenties niet meer dan 10% uitmaken van de totale mRNA populatie in de polysomen op 37°C. In dit opzicht is er een duidelijk verschil met D. melanogaster waar een meer stringent translatie effect in sommige van de cellijnen wordt waargenomen.

De heat shock loci en hun eiwitprodukten

In hoofdstuk V en VI zijn de resultaten beschreven aan de hand waarvan een correlatie gevonden kan worden tussen de heat shock loci en de produkten waarvoor zij coderen. De 20S RNA fractie bevat enerzijds sequenties die homoloog zijn aan locus 2-32A, 2-36A en 4-81B en codeert anderzijds voor de 70.000 en 67.000 dalton polypeptides. De 13S RNA fractie codeert voor de laag moleculaire eiwitten en hybridiseert met locus 4-85B en in enigermate met locus 2-32A. Aan de hand van

deze resultaten, in combinatie met de gegevens verkregen met de hybride-arrested translaties is komen vast te staan dat van elk van de heat shock genen, zichtbaar als puffs in polytene chromosomen, boodschapper RNA's worden afgelezen die coderen voor een van de heat shock eiwitten. Alleen locus 2-48B vormt in dit opzicht een uitzondering aangezien het merendeel van het afgelezen RNA de kern niet verlaat.
In hoofdstuk IV werd een correlatie gevonden tussen locus 2-36A en het 70.000 dalton eiwit. Bij nader inzien is deze conclusie misschien wat voorbarig geweest omdat er bij de interpretatie van de gegevens vanuit is gegaan dat de 67.000 eiwitband uit slechts een polypeptide zou bestaan. Onlangs is in ons laboratorium aangetoond dat deze band in feite minstens vijf polypeptides bevat die onderling kleine verschillen in isoelectrisch punt vertonen. Bovendien heeft peptide mapping uitgewezen dat drie van deze polypeptides een practisch identieke aminozuurvolgorde hebben terwijl de andere twee slechts een gedeeltelijke overeenkomst hiermee vertonen. Op grond hiervan zou men ook de twee RNA soorten, die voor deze polypeptides coderen, moeten kunnen onderscheiden. Inderdaad doet een dergelijke situatie zich voor bij locus 87A/C en 95D in D. melanogaster. Gecloneerde genoom fragmenten van deze twee loci in D. melanogaster hybridiseren in situ ook met de overeenkomstige heat shock loci in D. hydei, n.l. 2-32A en 2-36A. Daarnaast vertonen beiden echter ook een duidelijke kruishybridisatie zoals aangetoond is in hoofdstuk VII. De sequentie homologie tussen de RNA's afgelezen van deze loci geldt in ieder geval niet voor het 3' einde van de boodschappers, omdat recombinanten geconstrueerd via de cDNA procedure geen kruishybridisatie vertonen tussen 2-32A en 2-36A. Alhoewel het definitieve bewijs met behulp van hybride-arrested translatie geleverd zal moeten worden, lijkt het zeer waarschijnlijk dat locus 2-32A en 2-36A elk een aantal verwante boodschapper RNA's produceren die te samen voor de 67.000 dalton polypeptides coderen. De suggestie gedaan in hoofdstuk IV als zou locus 2-36A coderen voor het 70.000 dalton eiwit, is daarom niet juist. Onlangs is aangetoond dat locus 4-81B voor dit eiwit codeert. Dit locus bleek ook actief te zijn in controle weefselkweekcellen (hoofdstuk VI) hetgeen zou betekenen dat het eiwit waarvoor het codeert, aanwezig ook onder normale condities, een essentiële functie heeft in metabolisme of ontwikkeling. De concentratie van het 70.000 dalton polypeptide

in de originele fractie waarmee het antilichaam werd opgewekt, zal daarom voldoende hoog geweest zijn om antigeen te werken hetgeen de specifieke reactie van het antiserum met het 70.000 dalton polypeptide verklaar (fig. 2, hoofdstuk IV). Anderzijds had in dat geval ook een toename van de hoeveelheid zilverkorrels over locus 4-81B gevonden moeten worden. De korreldichtheden voor dit locus waren echter dermate laag, dat zij niet in de analyse betrokken werden. Resumerend, kan uit de hier beschreven experimenten geconcludeerd worden dat de door een heat shock geinduceerde genen boodschapper RNA's produceren die coderen voor de heat shock eiwitten, uitgezonderd locus 2-48B dat voornamelijk codeert voor kern specifiek RNA. Gecloneerde fragmenten van deze buitengewone kerntranscript zullen opheldering moeten brengen omtrent hun structuur en misschien ook omtrent hun functie in de heat shock response.

CURRICULUM VITAE

Paul Sondermeijer werd geboren op 24 november 1951 te Willemstad op de Nederlandse Antillen. De middelbare schoolopleiding werd gevolgd in Den Haag en Leiden en afgesloten met het behalen van het diploma HBS-B in 1969. Hierna schreef hij zich in voor de studie scheikundige technologie aan de TH Delft. Het kandidaatsexamen werd behaald in september 1973 waarna richting IV (biologie) voor het afstuderen werd gekozen. Tijdens deze periode was hij werkzaam in het laboratorium voor microbiologie (Prof. Dr. T.O. Wikén) en werd onderzoek gedaan o.l.v. Ir. H.Y. Steensma aan de defectieve bacteriofagen van B. subtilis. Tevens volgde hij de cursus industriële democratie en behaalde zijn lesbevoegdheid in de wis- en scheikunde. Zijn studie aan de TH werd in de zomer van 1975 afgesloten met het ingenieursdiploma. In augustus van hetzelfde jaar volgde de aanstelling bij de de afdeling Genetica van de Katholieke Universiteit Nijmegen. Onder de leiding van Dr. N.H. Lubsen wijdde hij zich aan de moleculaire genetica van het heat shock systeem in Drosophila hydei. De resultaten hiervan, in de vorm van een aantal publicaties, zijn voor het merendeel in dit proefschrift verwerkt.
Sinds 1 januari 1981 is hij werkzaam in Straatsburg bij Transgene S.A., een onderneming actief in het ontwikkelen van commercieel-industriële toepassingen van het DNA-recombinant onderzoek.

STELLINGEN

1. Uit de experimenten van Brandt en Milcarek kunnen nauwelijks harde conclusies getrokken worden omtrent het lot van het bij 25°C aanwezige poly(A^+) RNA tijdens een heat shock behandeling.

   Brandt, C. en Milcarek, C. (1980)
   Biochemistry 19, 6152 - 6158

2. De suggestie als zou de mate van transcriptie bij 25°C van een heat shock gen in Drosophila cellen vergelijkbaar zijn met die van een β-globine gen in een gedifferentieerde erythroide cel kan niet serieus genomen worden.

   Craig Findly, R. en Pederson, T. (1981)
   J. Cell. Biol. 88, 323 - 328

3. De conclusie van Oppermann et al. als zou het pp50 eiwit na transformatie met RSV eerder een cel dan virus specifieke herkomst hebben wordt onvoldoende door de door hen aangevoerde gegevens ondersteund.

   Oppermann, H., Levinson, W. en Bishop, J.M. (1981)
   Proc. Natl. Acad. Sci. USA 78, 1067 - 1071

4. Uit de experimenten van Levinson et al. zou geconcludeerd moeten worden dat het na transformatie met RSV gevonden eiwit $pp52^{scr}$ in feite de actieve transformerende component is met het nauwverwante $pp60^{scr}$ als mogelijke voorloper.

   Levinson, A.D., Courtneidge, S.A. en Bishop, J.M. (1981)
   Proc. Natl. Acad. Sci. USA 78, 1624 - 1628

5. Naast het door Neufeld et al. voorgestelde "secretion-recapture" mechanisme voor het transport van lysosomale enzymen in fibroblasten, is ook een direkt intracellulair transport naar de lysosomen aanwezig.

   Neufeld, E.F., Sando, G.N., Garvin, A.J. en Rome, L.H. (1977)

J. Supramolecular Structure 6, 95 - 101
von Figura, K. en Weber, E. (1978)
Biochem. J. 176, 943 - 950

6. Bij het onderzoek naar de factoren die een rol spelen in de gen expressie bij eukaryoten zal terdege rekening gehouden moeten worden met het drastische effect van subtiele verschillen in de sequentie die bepalend kunnen zijn voor het feit of een gen al dan niet functioneel actief is.

   Gannon, F., Jeltsch, J.M. en Perrin, F. (1980)
   Nucleic Acids Res. 8, 4405 - 4421

7. Dankzij elegante technieken zoals in vitro recombinatie, mini-deletie constructie en S_1-mapping, heeft men op dit moment voor eukaryoten een beter inzicht in de promotor structuur dan voor prokaryoten.

   Corden, J., Wasylyk, B., Buchwalder, A., Brison, O. en Chambon, P. (1980)
   Science 209, 1406 - 1414
   Dierks, P., van Ooyen, A., Mantei, N. en Weissmann, C. (1981)
   Proc. Natl. Acad. Sci. USA 78, 1411 - 1415

8. De al enige tijd durende strijd omtrent de chromosoom localisatie van het β-interferon gen zal waarschijnlijk in het voordeel van een ieder beslist worden nu gebleken is dat er van dit type meerdere gerelateerde species voor kunnen komen.

   Sehgal, P.B., et al. (1981)
   Proc. Natl. Acad. Sci. USA, in druk

9. Ondanks het feit dat in de door Taniguchi et al. beschreven vector aan schijnbaar alle voorwaarden is voldaan om tot maximale expressie te komen, mag het resultaat in het geval van interferon teleurstellend genoemd worden.

   Taniguchi, T., Guarente, L., Roberts, T.M., Kimelman, D., Douhan, J. en Ptashne, M. (1980)

Proc. Natl. Acad. Sci. USA, 77, 5230 - 5233

10. Het gebruik van B. subtilis als "veilige" gastheer voor genetische manipulaties zal vooralsnog beperkt blijven gezien de instabiliteit van recombinant plasmiden in dit organisme.

    Uhlen, M., Flock, J. en Philipson, L. (1981)
    Plasmid 5, 161 - 169

11. Het bestaansrecht van commerciële bedrijven op het gebied van het recombinant-onderzoek zou niet enkel vanuit het standpunt van winstoogmerk bezien moeten worden.

12. In het kader van een verantwoord aanstellingsbeleid lijkt het de overweging waard om ook voor hoogleraren een proeftijd van een jaar in te stellen.

P.J.A. Sondermeyer

Straatsburg, 1 augustus 1981