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Developmental and Functional Analysis of *Jonah* Gene Expression

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The *Jonah* genes are expressed twice in development: *Jonah* RNA is detected during all larval stages, disappears at the end of the third larval instar, and then reappears shortly after eclosion, in the adult midgut. Construction and analysis of *Jonah* cDNA clones reveals that multiple *Jonah* genes are transcribed; cDNA clones deriving from at least four different clusters of *Jonah* genes have been identified. In at least one case, multiple genes in a cluster are transcribed, and one cluster is found to be transcribed both in larvae and adults. Evidence that different *Jonah* genes are under different control with respect to both spatial and temporal patterns of expression has been provided. *Jonah* RNA encodes a 28-kDa translation product or products for which we consider a possible function. *Jonah* RNA of constant length is found to be conserved in all strains of *Drosophila melanogaster* examined, *Jonah* genes are found at a minimum of three common chromosomal sites in all of seven *D. melanogaster* strains examined, and multiple *Jonah* genes are found in other *Drosophila* species.

INTRODUCTION

In the preceding article (Carlson and Hogness, 1985) we described the chromosomal arrangement and structural variation of the *Jonah* genes of *Drosophila melanogaster*. Our analysis revealed a complex family consisting of approximately 20 genes distributed in small clusters at eight or more widely dispersed chromosomal sites. Gene clusters differ both in the number of genes per cluster and in the topography of clustering, and individual genes exhibit heterogeneity in structure. In the third-instar larva the *Jonah* genes are abundantly expressed as transcripts of a single size class, located exclusively in the midgut.

In the present article we extend our description of the expression of the *Jonah* genes and show that the family is complex in its pattern of expression as well as in its organization. We begin by examining the developmental regulation of *Jonah* transcription and find that the family is expressed twice in development: *Jonah* RNA is detected during all larval stages, disappears at the end of the third larval instar, and then reappears in adults, again in the midgut. In order to examine the pattern of expression of individual *Jonah* genes, we exploit the structural heterogeneity among *Jonah* genes by constructing cDNA clones, analyzing their structure, and correlating *Jonah* cDNA clones with particular members of the gene family. The structural variation is also used to provide evidence that different *Jonah* genes are under different control with respect to both spatial and temporal specificity of expression.

We consider finally the function which this elaborate gene system has evolved to fulfill. We investigate first the function of *Jonah* RNA and find that it encodes a translation product or products of 28 kDa. The functional importance of certain characteristics of the family is addressed; we adopt a comparative approach based on the expectation that features of the family's organization and expression essential to its function should be maintained in evolution. *Drosophila* of other species and strains are examined to determine the extent to which the presence of *Jonah* RNA, the length of *Jonah* RNA, the existence of multiple *Jonah* genes, and the chromosomal location of *Jonah* genes are maintained. We conclude by drawing certain relationships between patterns of organization and expression of the *Jonah* genes and by considering a possible function for their products.

MATERIALS AND METHODS

Materials

All materials were as described in Carlson and Hogness (1985) except that terminal transferase was obtained from R. Ratliff, and [^{35}S]-methionine was purchased from New England Nuclear.

Unless otherwise indicated, *D. melanogaster* were obtained and maintained as described in Carlson and Hogness (1985). *D. melanogaster* of other strains derived from stocks obtained from S. Beckendorf (University of California, Berkeley) in the case of the experiment shown in Fig. 9, or from E. Meyerowitz (California

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Institute of Technology) in the experiment shown in Table 2. Flies other than *D. melanogaster* were obtained by M. Akam (Cambridge University).

Preparation of RNA

RNA was generally prepared by phenol extraction. In some experiments the organisms were flash frozen in liquid nitrogen and stored at −20°C prior to extraction. When extracting RNA from single hand-isolated organs, tRNA was added as carrier. Tissue culture cell RNA was extracted from the Kc_0 line (Lis *et al.*, 1978; Eschalier and Ohanessian, 1970) by C. Parker; embryonic RNA was a gift of R. Lifton. RNA used in the experiment shown in Fig. 9 was prepared as described in Muskavitch (1980). RNA used in the hybrid selection experiment was prepared by a method similar to that of Chirgwin *et al.* (1979). Poly(A)$^+$ RNA was isolated by passage over oligo(dT)-cellulose.

The midgut tissue used as a source of RNA in Fig. 8b was prepared using a variation of a technique described by Wolfner (1980): late third-instar larvae, immersed in aerated Robb's insect medium (Robb, 1969), were crushed between metal rollers and the grindate was filtered through a Nitex 600 screen; the material trapped on the screen is enriched in large pieces of gut and cuticle, and pieces of midgut are hand-picked under a dissecting microscope.

Polysomes were isolated with minor modifications of the method of McKenzie *et al.* (1975).

Manipulation of RNA and DNA

RNA was denatured and fractionated by electrophoresis as described in Carmichael and McMaster (1980). RNA was transferred either to DBM paper (Alwine *et al.*, 1977) or to ATP paper (Seed, 1982) in 100 m*M* NaAc (pH 4.0) buffer. The paper was prehybridized at 42°C for at least 4 hr in 20 ml of 50% formamide, 5× SSCP (1× SSCP = 120 m*M* NaCl, 15 m*M* Na_3citrate, 20 m*M* Na phosphate (pH 6.8)), 0.5 mg/ml carrier DNA, 0.1% SDS, 1× Denhardt's solution (0.02% BSA, 0.02% Ficoll, 0.02% polyvinylpyrrolidone) (Denhardt, 1966), and 1% glycine.

Hybridization to filters was conducted at 42°C for 12-48 hr in 50% formamide, 5× SSCP, 1 mg/ml carrier DNA, 0.5 mg/ml poly(A), and 1× Denhardt's solution. The probe was heated at 80°C for 2-5 min in the hybridization buffer before use. Following hybridization, the filters were washed at 42-45°C for 3 hr with four changes in 50% formamide, 5× SSC (1× SSC = 150 m*M* NaCl, 15 m*M* Na_3citrate). Filters were exposed to Kodak XR-5 X-ray film at −70°C with a Dupont Cronex Lightning-Plus intensifying screen.

Filters that had been hybridized with one probe were washed under denaturing conditions before being hybridized with another. The radioactivity on the filter was allowed to decay over the course of several half-lives, and then the filter was washed 3× 30 min in 95% formamide, 20 m*M* Tris (pH 8.0), 0.1% SDS, and 5 m*M* EDTA in a sealed plastic bag at 65°C on a rocking platform. The filters were exposed to X-ray film following this treatment prior to hybridization with the next probe.

Digestion, electrophoresis, transfer, labeling, and hybridization of DNA were as described in Carlson and Hogness (1985), except that in the analysis of other species, DNA was transferred to ATP paper (Seed, 1982) and the filter washed in 0.1% SDS, 2× SSC at 45°C for 4 hr.

Recombinant DNA

The cDNA clones pkdm26H2, pkdm8G8, adm135A8, and adm126B4 were constructed as described by Wolfner (1980); pkdm26H2 and pkdm8G8 were among a set of clones constructed by insertion of cDNA segments into the *Bam*HI site of the plasmid pSC105 (Cohen *et al.*, 1973) by $(dA)_n \cdot (dT)_n$ joints; adm135A8 and adm126B4 were among a set of clones constructed by insertion of cDNA segments into the *Pst*I site of the plasmid pBR322 (Bolivar *et al.*, 1977) by $(dG)_n \cdot (dC)_n$ joints. The other *Jonah* cDNA clones were constructed as detailed below.

The RNA used in cDNA synthesis, extracted in the absence of diethylpyrocarbonate, was passed through an oligo(dT)-cellulose column in 0.5 *M* NaCl, 10m*M* Tris (pH 7.4), 1 m*M* EDTA, and poly(A)$^+$ RNA was eluted in H_2O. Following ethanol precipitation, the RNA was used as a template for the synthesis of cDNA in a reaction containing 5 μg/ml oligo(dT) as primer; reaction conditions were essentially those of Wahl *et al.* (1979). The reaction was terminated and the RNA-DNA duplexes were denatured by boiling for 1.5 min. The second-strand synthesis reaction was performed at 12°C for approximately 5 hr following the addition of 50 m*M* Hepes (pH 7.0), 50 m*M* KCl, 100 μ*M* each of dATP, dGTP, and dTTP, 50 μ*M* of dCTP, and 4 μg of DNA polymerase I, in a total volume of approximately 50 μl.

The cDNA molecules were digested with 500 units of S1 for 30 min at 37°C following the addition of a 5× excess of a buffer containing 30 m*M* NaAc (pH 4.4), 250 m*M* NaCl, and 1 m*M* $ZnCl_2$. The reaction was terminated by the addition of 150 m*M* Tris (pH 7.5) and 1% diethylpyrocarbonate. Following a 30 min incubation at 37°C, the cDNA was ethanol-precipitated in the presence of tRNA carrier. The cDNA was

fractionated by size on a Bio-Gel A50 column; fractions were electrophoresed on a 1.5% agarose gel, which was dried and exposed to X-ray film. Fractions containing cDNA segments of mean length greater than 500 bp were pooled and ethanol precipitated.

The double-stranded cDNA was tailed with $(dC)_n$ using terminal transferase essentially as described by Wahl *et al.* (1979). The tailed cDNA was extracted 2× with phenol and 3× with ether, and the aqueous phase was placed in a vacuum to remove residual ether. Poly(dG)-tailed pBR322 was prepared by digesting with *PstI*, extracting the reaction mix with phenol and then ether, precipitating with ethanol, and tailing with $(dG)_n$ essentially as described by Wahl *et al.* (1979).

The $(dC)_n$-tailed cDNA and the $(dG)_n$-tailed pBR322 were annealed in 100 m*M* NaCl, 10 m*M* Tris (7.5), and 0.2 m*M* EDTA by heating at 64°C for approximately 3 min, incubating at 42° for 2 hr, and then allowing the mixture to cool to room temperature over a period of several hours. The annealed mixture was used to transform *Escherichia coli* strain HB101 as described by Davis *et al.* (1980). Transformants were picked into arrays on selective plates, replica-plated, and screened essentially as described by Grunstein and Hogness (1975).

Genomic clones and subclones were constructed and isolated as described in Carlson and Hogness (1985). The genomic subclone aDm3201 contains the 1.8-kb *Hin*dIII fragment of λbDm2306 inserted into the *Hin*dIII site of pBR322, and thus contains *Jon99Cβi* or *ii*, as does aDm2353 (see Carlson and Hogness, 1985).

DNA of the plasmids pkdm26H2, pkdm8G8, adm-135A8, and adm126B4 were gifts of M. Wolfner and G. Guild (Wolfner, 1980). For other plasmids, the isolation method of Davis *et al.* (1980) was used. The purified 1.8-kb *Hin*dIII fragment deriving from aDm3201 was prepared on neutral sucrose gradients by M. Akam (Cambridge University).

Phage DNA was prepared as described in Carlson and Hogness (1985).

Hybrid Selection

Hybrid selection was performed by a procedure similar to those of Ricciardi *et al.* (1979) and Cleveland *et al.* (1980). Cloned DNA was immobilized on a nitrocellulose filter with minor modifications of the method of Kafatos *et al.* (1979). The filter was prehybridized at 37°C for 1 hr in the hybridization buffer, which consisted of 50% deionized formamide, 400 m*M* NaCl, 100 m*M* Pipes (pH 7.0), 5 m*M* EDTA, 0.1% SDS. Approximately 45 μg of poly(A)$^+$ RNA, extracted from late third-instar larvae, was treated at 68°C for 2 min and then allowed to hybridize to the filter at 37°C in a total volume of 200 μl for approximately 2 hr. The filter was washed 10× 1 ml in hybridization buffer minus SDS for a total of 1.5 hr. RNA was eluted from the filter in 200 μl of 70% formamide, 50 m*M* Pipes (pH 7.0), and 10 μg yeast tRNA carrier at 65°C for 5 min. The eluate was spun to remove debris, and the supernatant was precipitated three times with ethanol, dried, and resuspended in water.

In Vitro Translation

A nuclease-treated rabbit reticulocyte lysate, purchased from Amersham, was used for *in vitro* translation. RNA samples were heated at 68°C for 2–4 min before translation, the translation reactions were allowed to proceed at 30°C for 60–75 min, and the reactions were terminated by treatment with 30 ng of RNase A for 20 min and 50 ng of DNase I for 10 min. Following the addition of 2% SDS, 40 m*M* DTT, or 1% β-mercaptoethanol, 60 m*M* Tris-HCl (pH 6.8), 0.5 m*M* EDTA, 10% glycerol, and bromophenol blue, the translation mixes were heated at 90°C for 1 min before being loaded on 15% SDS-polyacrylamide gels (Laemmli, 1970). Proteins containing [^{35}S]-methionine were visualized by fluorography (Bonner and Laskey, 1974).

RESULTS

Temporal Specificity of Expression

In the accompanying article (Carlson and Hogness, 1985) we defined the *Jonah* gene family on the basis of homology to a cloned cDNA segment, dm26H2, which derives from poly(A)$^+$ RNA isolated from third-instar larvae. In order to determine whether the *Jonah* genes are expressed at developmental times other than the third larval instar, we extracted RNA from whole animals at a series of developmental stages, a large number of organisms ($N > 35$) being used for each RNA sample. Figure 1 shows an autoradiogram of a filter containing electrophoretically fractionated RNA from 14 developmental stages, and from a line of Kc_0 cells, after hybridization with the reference cDNA clone. While no *Jonah* RNA was detected in embryos, it was found in larvae of all three instars. The *Jonah* RNA level declines sharply at the end of the third instar, and is not detectable at any of several times examined throughout the pupal stage. However, *Jonah* RNA reappears in adults. The RNA is of constant size (0.9 kb) in all larval tracks and in the adult track. Kc_0 cells contain no detectable *Jonah* RNA.

Further experiments have shown that *Jonah* RNA does not make its appearance in adults until several hours after emerging from the pupal case; we have not found it in flies examined 0–1 hr or 7 hr ± 30 min after

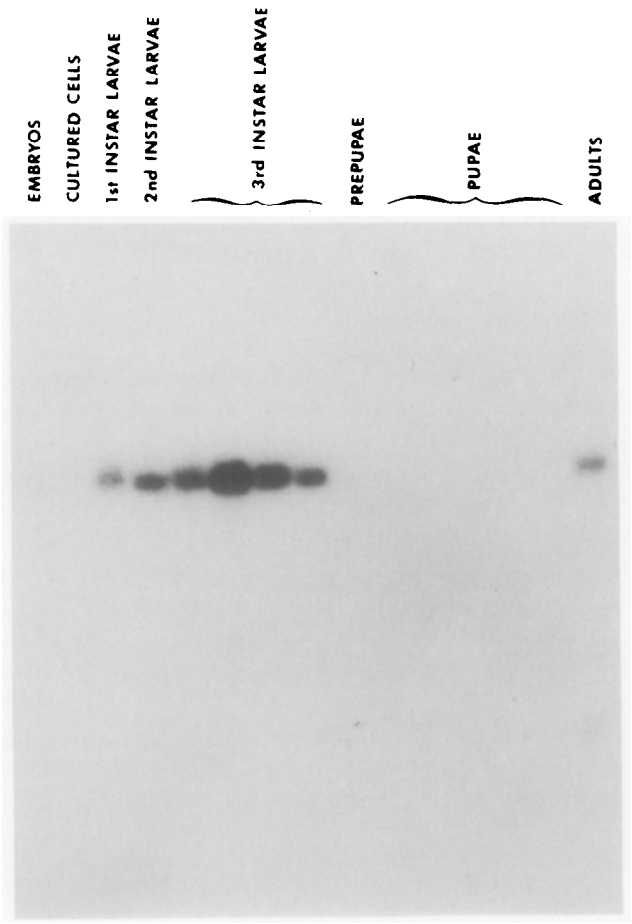

FIG. 1. Developmental profile of *Jonah* RNA. Each track contains approximately 15 μg of total RNA from organisms at the indicated developmental stages. RNA was extracted, electrophoresed, transferred to DBM paper, and hybridized with a ^{32}P-labeled *Jonah* cDNA probe as described under Materials and Methods. Of the four third-instar tracks, the one on the left represents larvae which were burrowing in the culture food, and the other three tracks represent larvae at the climbing stage, isolated from the walls of the cage at successively later times. Pupae were approximately (left to right): 5.5, 6.5, 7.5, 9, 9 day (pharate adults). See text for experiments with adults of various ages.

eclosion, but it is detectable in flies 12 hr ± 30 min after eclosion. *Jonah* RNA remains present throughout adult life in flies of both sexes; we have detected the RNA in flies of varying ages up to and including 60 days. Female flies contain significantly more *Jonah* RNA than males; however, females contain more total RNA, and we have not compared levels of *Jonah* RNA per microgram of total RNA.

Tissue-Specificity of Expression

The dissection experiment shown in Fig. 1 of the preceding article (Carlson and Hogness, 1985) identified the midgut as the source of *Jonah* RNA in third-instar larvae. This localization of *Jonah* RNA to the larval midgut was confirmed by *in situ* hybridization to RNA in tissue sections (Akam and Carlson, in press). Inspection of labeled midgut cells reveals that *Jonah* RNA is predominantly located in the cytoplasm.

The identification of *Jonah* RNA in adults raises the question of the tissue specificity of this adult RNA: the larval midgut, the source of larval *Jonah* RNA, is histolyzed at the end of the third instar. The larval midgut is replaced, however, by an adult midgut, which develops during the pupal period. The adult midgut derives from small nests of imaginal cells which are located between the large epithelial cells of the larval midgut and the external basement membrane and which escape histolysis (Bodenstein, 1965). Figure 2 shows that this adult midgut is the source of adult *Jonah* RNA; the RNA is not detectable in other tissues.

Genetic Specificity of Expression

Experiments described above have analyzed the expression of the *Jonah* gene family as a whole, by measuring levels of the entire population of *Jonah* RNA. In order to identify individual transcripts, we have prepared and analyzed *Jonah* cDNA clones. Structural analysis of the gene family, described in Carlson and Hogness (1985) and below, has revealed extensive restriction site heterogeneities among different genes and has shown that certain restriction site patterns are unique to specific cloned genes. We therefore anticipated that cDNA clones would contain diagnostic restriction patterns that could be correlated with those of individual *Jonah* genes.

Poly(A)$^+$ RNA was prepared from third-instar larvae and from adults and, in separate experiments, was used as the template for cDNA synthesis. Double-stranded cDNA was fractionated by size, and fractions containing cDNA of mean length greater than approximately 500 bp were pooled and cloned by $(dG)_n \cdot (dC)_n$ tailing into the *Pst*I site of pBR322 (Bolivar *et al.*, 1977). Approximately 275 larval and 560 adult cDNA clones were screened by the method of Grunstein and Hogness (1975), using as probe the 1.8-kb *Hin*dIII fragment which contains the *Jon99Cβi* or *ii* genes, and which exhibits a restriction map corresponding to that of dm26H2, the reference cDNA segment. (See Carlson and Hogness, 1985, for *Jonah* gene nomenclature). Ten positives were obtained, of which six, three larval and three adult, have been correlated with specific genomic genes. These six, along with four other cDNA clones prepared in separate cloning experiments (Wolfner, 1980), yield insert-specific restriction fragments which

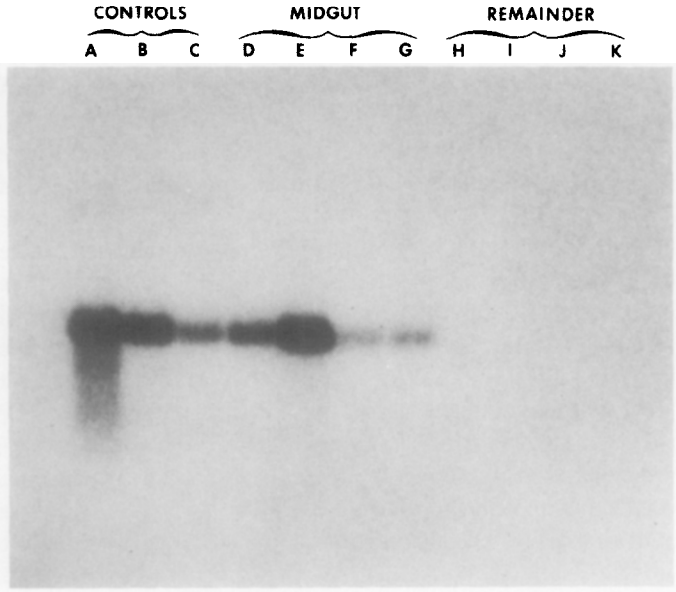

FIG. 2. Tissue-specificity of *Jonah* RNA in adults. Four individual adult organisms, two male and two female, were hand-dissected in Robb's medium. The midgut was isolated by severing the esophagus immediately anterior to the cardia and by severing the ventriculus in the pyloric region, immediately anterior to the point at which the Malpighian tubules join. RNA was extracted separately from the isolated midgut and all remaining tissues of each dissected organism as well as from two control organisms, surgically opened but not dissected. RNA was extracted, electrophoresed, transferred to ATP paper, and hybridized with ^{32}P-labeled pkdm26H2 as described under Materials and Methods. Lane A contains 3 μg of total third-instar larval RNA; lanes B and C contain RNA from undissected female and male controls, respectively; lanes D and E contain RNA extracted from female midguts; lanes F and G contain RNA extracted from male midguts; lanes H and I contain RNA from remaining female tissue; lanes J and K contain RNA from remaining male tissue.

match in length restriction fragments peculiar to specific *Jonah* genes, with an exception discussed below.

Figure 3 provides examples of the type of analysis used in correlating cDNA clones with specific genomic genes. Two cDNA clones, digested with pairs of restriction enzymes, are electrophoresed adjacent to those genomic subclones which, when digested with the same pairs of enzymes, yield the corresponding diagnostic fragments. These diagnostic fragments, specific to the inserts of the cDNA clones, are indicated by arrows. The chromosomal site from which each genomic subclone derives is indicated above the appropriate tracks. Maps representing inserts of the 10 cDNA clones and of the genomic subclones to which they correspond are shown in Fig. 4.

This method of assigning cDNA clones to particular genomic genes, based on the comigration of corresponding restriction fragments, is limited in two respects. First, if an intervening sequence exists within a diagnostic genomic restriction fragment, the corresponding cDNA fragment will be smaller than its genomic correlate, and assignment may be difficult. This appears to be the case for two of the cDNA clones we have analyzed, adm135A8 and adm2372, but in the case of these clones the limitation has been overcome. These cDNA clones yield an *Ava*I-*Sal*I fragment of 500 bp, and there is no corresponding RNA-homologous fragment in any of our genomic *Jonah* clones (Carlson and Hogness, 1985, and unpublished results). There is, however, a 600-bp *Ava*I-*Sal*I fragment in the *Jon65Aiv* gene which is carried by the genomic subclone aDm2358. When adm135A8 is hybridized to total genomic DNA digested doubly with *Ava*I and *Sal*I, the band which hybridizes most intensely is 600 bp long, and there is no hybridizing genomic band 500 bp long; this 600-bp band comigrates with the *Ava*I-*Sal*I fragment in aDm2358 and derived from *Jon65Aiv*. We therefore conclude that adm135A8 derives from *Jon65Aiv*, which contains a 100-bp intron. No comparable evidence for

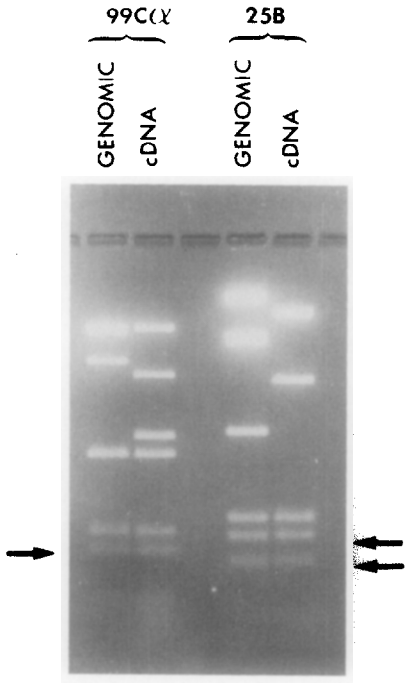

FIG. 3. Correlation of cDNA clones with *Jonah* genes. The four tracks, left to right, represent 1 μg of each of the following clones, digested with the indicated pairs of restriction endonucleases: aDm2359, *Bam*HI and *Bgl*I; adm2375, *Bam*HI and *Bgl*I; aDm2356, *Bam*HI and *Sal*I; adm2370, *Bam*HI and *Sal*I. The chromosomal sites listed above the tracks are those from which the cloned genomic DNA has been shown to derive by *in situ* hybridization (Carlson and Hogness, 1985). The arrows indicate those bands representing diagnostic restriction fragments contained entirely within the inserts of both the cDNA clones and the genomic clones. The sizes of these fragments are 200 bp in the case of the insert-specific fragment common to the left pair of tracks, and 220 and 180 bp for those common to the right pair of tracks.

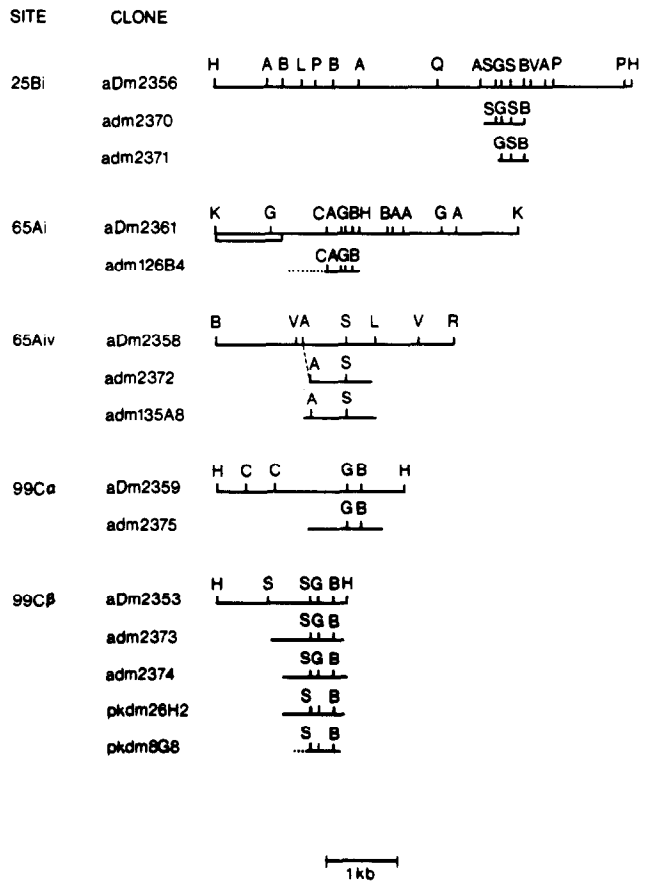

FIG. 4. Maps of *Jonah* cDNA clones and the corresponding *Jonah* genes. Restriction maps are shown for genomic Dm segments at the indicated chromosomal sites; corresponding cDNA dm segments are drawn below with maps of restriction sites found to correspond to genomic restriction sites. Only one genomic *Jonah* gene is shown for 99Cα and 99Cβ although one other gene at 99Cα and two other genes at 99Cβ share the same diagnostic pattern. Maps are oriented so that inferred 5' ends of *Jonah* genes are to the left. Horizontal dotted lines indicate uncertainty as to the positions of the 5' ends of dm126B4 and dm8G8 segments. The bar under the left end of the aDm2361 map indicates λ sequences (Carlson and Hogness, 1985). The dashed line connecting the *Ava*I sites of aDm2358 and adm2372 indicates that these sites correspond despite the differences in distance from the adjacent *Sal*I site (see text). We presume there are *Bgl*I sites at the positions of the unmarked vertical lines in pkdm26H2 and pkdm8G8, but this has not been tested. aDm2361 has not been mapped for L, P, Q, or V; C sites have not been mapped in aDm2359, aDm2353, aDm2361; V sites have not been mapped in aDm2359. A, *Ava*I; B, *Bam*HI; C, *Sac*I; G, *Bgl*I; H, *Hin*dIII; K, *Kpn*I; L, *Bgl*II; P, *Pvu*I; Q *Hpa*I; R, *Eco*RI; S, *Sal*I; V, *Pvu*II.

introns in the other *Jonah* genes shown in Fig. 4 was obtained.

A second limitation of our method is that it is conceivable that there exist other, uncharacterized *Jonah* genes in our Oregon-R stock which contain fragments identical in length to those we consider diagnostic of specific characterized *Jonah* genes. In view of these two limitations, we have used another method, *in situ* hybridization, to confirm our assignment of cDNA clones to chromosomal sites. All 10 cDNA clones show patterns of *in situ* hybridization consistent with the assignments based on restriction analysis. Table 1 lists the cDNA clones, the developmental stage from which each derives, and the genomic origin of each.

Table 1 shows that multiple *Jonah* genes are transcribed: a total of five distinguishable *Jonah* genes are represented, deriving from four different chromosomal arrangements. Table 1 reveals that two *Jonah* genes residing at the same site are both active at the same developmental stage: adm126B4 derives from *Jon65Ai;* adm135A8 and adm2372 derive from *Jon65Aiv,* approximately 11kb removed from *Jon65Ai,* as shown in Fig. 4 of Carlson and Hogness (1985). The two types of gene deriving from 99C, designated 99Cα and 99Cβ, represent allelic variants at the 99C site, as discussed in Carlson and Hogness (1985); both types of gene are expressed. As the restriction pattern diagnostic of 99Cα genes is shared by two genes in an inverted repeat, and that of 99Cβ by three clustered genes, we have not distinguished precisely which of the *Jonah* genes in each arrangement is the source of these cDNA clones. However, Table 1 shows that at two different times in development, at least one of the 99Cβ-type *Jonah* genes is active, since corresponding cDNA clones have been recovered from both larval and adult RNA.

Structural variation among different *Jonah* genes may be exploited in another way in order to compare the composition of *Jonah* RNA deriving from different sources. This approach reveals differences between *Jonah* RNA populations deriving from different parts

TABLE 1
Jonah cDNA CLONES AND THEIR GENOMIC ORIGINS

cDNA clone	Developmental stage of origin	Genomic origin: Determination by restriction analysis	Genomic origin: Major site by *in situ* hybridization
adm2370	Larval	25Bi	25B, 65A, 99C, 99F[a]
adm2371	Larval	25Bi	25B
adm2372	Larval	65Aiv	65A
adm135A8	Larval	65Aiv	65A
adm126B4	Larval	65Ai	65A
pkdm26H2	Larval	99Cβ	99C
pkdm8G8	Larval	99Cβ	99C
adm2373	Adult	99Cβ	99C
adm2374	Adult	99Cβ	99C
adm2375	Adult	99Cα	99C

[a] Four sites are listed as all were of comparable intensity; a single major site could not be identified.

of the midgut, and between *Jonah* RNA populations deriving from different developmental stages; both the tissue-specific and the developmental time-specific differences are substantiated by two types of experiment.

Figure 5 reveals differences between *Jonah* RNA populations isolated from two different parts of the midgut. Figure 5a represents the same hybridization shown in Fig. 1 of the accompanying article (Carlson and Hogness, 1985), which shows hybridization of a 99Cβ-type probe to a variety of RNA preparations deriving from various organs of an individual third-instar larva; it is reproduced in part here to show the contrast with Fig. 5b, which represents the same filter hybridized under identical conditions with a *Jon65Aiv Jonah* probe. A striking difference is observed between the two autoradiograms: *Jon65Aiv*, but not *Jon99Cβ*, hybridizes to a track containing RNA extracted from the foregut and the anterior tip of the midgut, including the proventriculus, gastric caecae, and stomach. (See Fig. 6 for diagram of gut.)

The same results are obtained when the experiment is performed at higher resolution, using another *Jon99Cβ* probe and the *Jon65Aiv* probe in *in situ* hybridizations to RNA in tissue sections of third-instar larvae (Akam and Carlson, in press). As schematized in Fig. 6, the *Jon99Cβ* probe labels the most anterior part of the posterior midgut. *Jon65Aiv*, on the other hand, labels two other regions: one region includes the posterior part of the middle midgut; the other extends through most of the anterior midgut. The two probes were allowed to hybridize with different sections of the same individual larvae, ensuring that the results do not merely reflect differences among individual larvae.

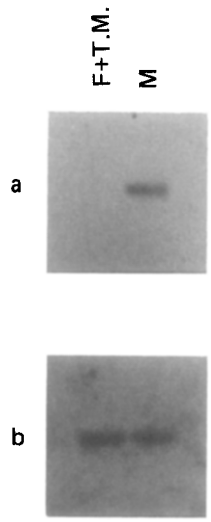

FIG. 5. Differences in tissue-specificity of filter hybridization between two *Jonah* cDNA clones. (a and b) Show the same filter hybridized sequentially with pkdm26H2 (a) and adm135A8 (b) under identical conditions, as described under Materials and Methods. The filter is the same as that shown in Fig. 1 of Carlson and Hogness (1985), where its preparation is described. F + T.M. = Foregut plus tip of anterior midgut; M = midgut.

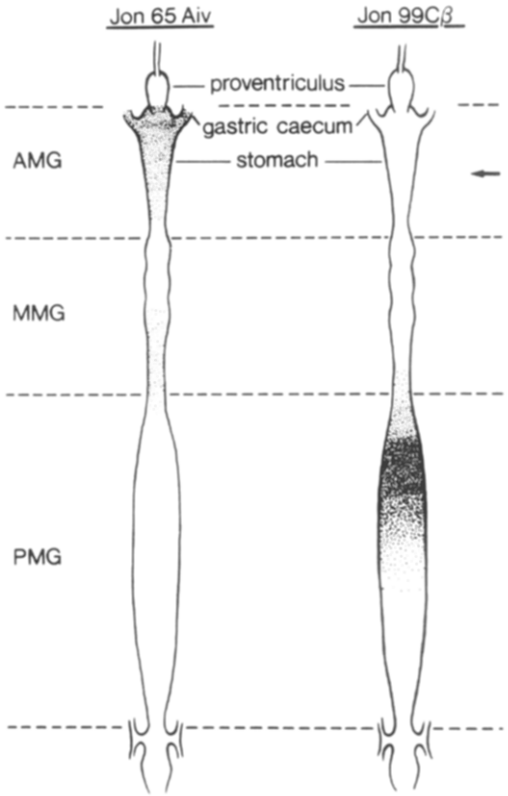

FIG. 6. Distribution of *Jonah* RNA in the larval gut. (Adapted from Akam and Carlson, in press). Stippled areas show regions of gut labeled with aDm3201 sequences (*Jon99Cβ*) or adm135A8 sequences (*Jon65Aiv*). Divisions between anterior midgut (AMG), middle midgut (MMG), and posterior midgut (PMG) are drawn as described in Akam and Carlson (in preparation). The arrow indicates the approximate position at which the midgut was severed in the dissection experiment shown in Fig. 5. Some cells in the vicinity of the MMG/PMG border label with both probes.

These experiments indicate that the composition of *Jonah* RNA in the anterior midgut clearly differs from that of the posterior midgut: *Jonah* RNA from the former region bears much stronger homology to *Jon65Aiv* than *Jonah* RNA from the latter region. The simplest explanation of these spatial differences in the steady-state composition of *Jonah* RNA is that there are differences in the spatial pattern of expression of individual *Jonah* genes, or in other words, that different *Jonah* genes are under different spatial control.

Different *Jonah* genes are also apparently under different temporal control, and this conclusion is again based on two types of experiment. We have shown above that the *Jonah* genes are active twice during development, once during larval stages and again during

the adult stage. As a preliminary means of investigating whether different genes are differentially expressed at different times in development, we isolated poly(A)$^+$ RNA from third-instar larvae and adults, and prepared oligo(dT)-primed ^{32}P-labeled cDNA from each. Cloned genomic DNA representing each of the seven characterized *Jonah* gene clusters was digested separately with restriction enzymes, divided into two equal aliquots, and fractionated on two identical agarose gels. The DNA in the two gels was then transferred in parallel to nitrocellulose filters and hybridized under identical conditions with ^{32}P-labeled cDNA from the two developmental stages. Even under our standard conditions of hybridization and washing, the hybridization patterns obtained with the two probes showed differences in the labeling of certain bands (data not shown). In particular, the larval cDNA showed significantly greater hybridization than adult cDNA to genomic fragments including the pair of *Jonah* genes deriving from 66C.

These results, suggesting that larval *Jonah* RNA and adult *Jonah* RNA can be distinguished on the basis of the extent of their homology with the *Jonah* genes at 66C, were confirmed by what is essentially the converse experiment, shown in Fig. 7. In this experiment, instead of hybridizing probes prepared from transcripts to immobilized genomic DNA, probes prepared from genomic DNA are hybridized to immobilized transcripts.

Figure 7 shows two autoradiograms of the same filter hybridized sequentially with different probes. The filter is the same as that shown in Fig. 1 and contains RNA extracted from a series of 14 developmental stages. Figure 7a shows the pattern obtained when ^{32}P-labeled λbDm2311, containing *Jon25Bi*, *Jon25Bii*, and *Jon25Biii*, is hybridized to the filter; as is the case with the reference cDNA clone pkdm26H2, hybridization to both larval and adult RNA is observed. However, Fig. 7b shows the pattern obtained when λbDm2307, the genomic clone carrying *Jon66Ci* and *Jon66Cii*, is used as probe. Although the larval tracks are clearly labeled, no detectable hybridization to the adult track is seen. The filter has also been probed with nick-translated λbDm2305, containing *Jon65Ai*, *ii*, *iii*, and *iv*, genes; this probe also shows homology to the adult track as well as the larval tracks.

The Identification of a Jonah Gene Product by in Vitro Translation

Several lines of evidence suggested that *Jonah* RNA encodes protein. First, *Jonah* RNA is present predominantly in the cytoplasm, as revealed directly by hybridization of cloned *Jonah* probes to RNA in tissue sections (Akam and Carlson, in press). Second, *Jonah* RNA binds to oligo(dT)-cellulose, indicating that it is polyadenylated. Third, cytoplasmic *Jonah* RNA cosediments with polysomes on sucrose gradients, as would be expected for a message bound to polysomes (data not shown).

Jonah RNA is very abundant. A rough measure of *Jonah* RNA abundance is provided by the frequency at which *Jonah* cDNA clones were isolated in the cDNA cloning experiments: 4 of approximately 275 cDNA clones constructed from third-instar larval RNA and 6 of approximately 560 cDNA clones constructed from adult RNA were identified as *Jonah* cDNA clones. The poly(A)$^+$ RNA used in these constructions was isolated from whole organisms. If we assume larval midgut RNA constitutes 1/10th of the RNA of the entire larva, a rough figure based on the intensities of fluorescence of ethidium bromide-bound RNA, then cDNA frequencies indicate that *Jonah* RNA constitutes as much as 10% of midgut poly(A)$^+$ RNA. If *Jonah* RNA encodes protein, as suggested above and shown below, then it would not be surprising if the protein it encodes were reasonably abundant.

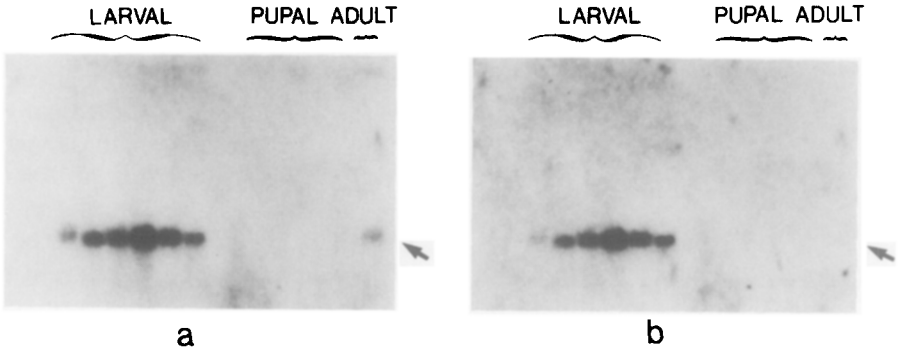

FIG. 7. A *Jon25B* probe and a *Jon66C* probe give different patterns when hybridized to RNA from multiple developmental stages. A DBM filter, the same as that shown in Fig. 1, was hybridized sequentially with (a) λbDm2311 and (b) λbDm2307, as described under Materials and Methods. The filter was prepared as described in the legend to Fig. 1. The arrow marks the position of *Jonah* RNA in the adult track.

The putative coding function of *Jonah* RNA has been investigated using the techniques of hybrid selection (Ricciardi *et al.*, 1979, Cleveland *et al.*, 1980) and *in vitro* translation. Approximately 45 μg of poly(A)$^+$ RNA extracted from third-instar larvae was allowed to hybridize to a nitrocellulose filter containing linearized DNA corresponding to six *Jonah* genes, of which four have been shown above to be expressed: the cDNA clones adm2372 and adm126B4, representing *Jon65Ai* and *Jon65Aiv;* the cDNA clones adm2373 and adm2375, representing 99Cα and 99Cβ; and aDm2356, a genomic subclone containing *Jon25Bii* and *Jon25Biii.* If *Jonah* RNA represents approximately 1% of organismal poly(A)$^+$, then the homologous DNA was in approximately 4× excess over the *Jonah* RNA. Eluted RNA was translated in a rabbit reticulocyte *in vitro* translation system, and the translation products were analyzed by SDS-polyacrylamide gel electrophoresis.

Figure 8A shows the results of this experiment. Track 1 shows the translation products of 0.3 μg of the total poly(A)$^+$ RNA ("organismal RNA") used in the hybridization; track 2 shows the translation products of one-half of the hybrid-selected RNA; track 3 is a control in which no RNA was added to the translation reaction. Given the relative amounts of RNA used in these translation reactions, one might expect the level of translation product of a hybrid-selected RNA to be enriched in the hybrid-selected RNA track by a factor of (1/2)(45 μg)/0.3 μg = 75 over the level of the corresponding translation product in the organismal track; this calculation assumes that the selected RNA is hybridized and eluted quantitatively and that the amount of its translation product is a linear function of the amount of that RNA added to the translation reaction.

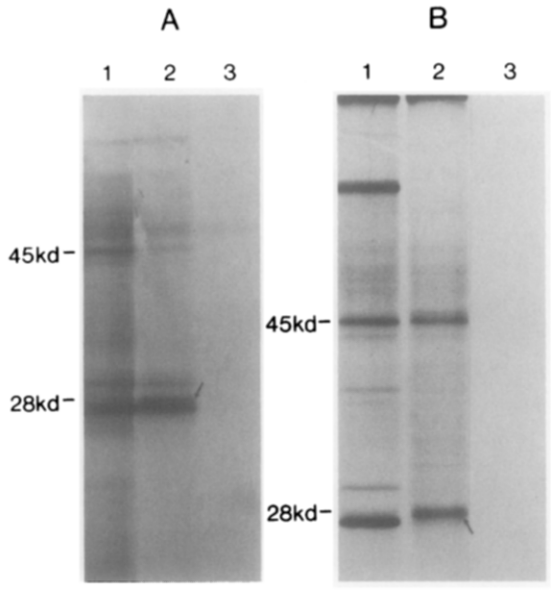

FIG. 8. *In Vitro* translation of hybrid-selected *Jonah* RNA, organismal RNA, and midgut RNA. Extraction of RNA, hybrid selection, and translation were as described under Materials and Methods. (A) Translations of (1) 0.3 μg organismal RNA, (2) hybrid-selected RNA, (3) no exogenous RNA. The arrow indicates the position of the translation product whose relative intensity is markedly enhanced following the hybrid selection procedure; the arrow points to the upper of two adjacent bands. The size of this product was determined in a separate experiment by comparison to φX174 protein standards. (B) Translations of (1) 5 μg organismal RNA, (2) 5 μg midgut-specific RNA, (3) no exogenous RNA. The arrow marks the expected position of *Jonah* translation products by analogy to (A).

The hybrid-selected RNA track in Fig. 8A contains several bands, but only one of them, indicated by the arrow, is more intense than the comigrating band in the organismal RNA track. The position of the band is that expected for a protein of 28,000 Da. We tentatively designate this band as a *Jonah* gene product and provide more evidence below that this is indeed the case. The other bands visible in the eluted RNA track are unlikely to derive from additional *Jonah* gene products. One of these bands is also present in the negative control, and the others may represent RNA species which bound nonspecifically to the filter: all are among the most abundant bands in the organismal RNA track, and are therefore precisely those bands one would expect to see if nonspecific binding were occurring. The above calculation predicted that a hybrid-selected band should be present at substantially greater intensity in the hybrid-selected RNA track than in the organismal RNA track, a condition which obtains only for the band designated by the arrow.

The experiment shown in Fig. 8A was performed with RNA extracted from whole organisms, and the identified *Jonah* gene product is only a relatively minor band among the translation products of organismal poly(A)$^+$ RNA. If our identification of the *Jonah* gene product is correct, then this same band should appear in a translation of RNA extracted from purified midgut tissue, and, furthermore, given that *Jonah* RNA is very abundant in the midgut, the *Jonah* RNA translation product might be expected to be among the most abundant translation products of the midgut. Both of these predictions are confirmed by Fig. 8B, which shows translations of organismal and isolated midgut RNA. There is indeed a band in the midgut track which migrates at the same relative position as the *Jonah* product identified in Fig. 8A; moreover, it is the most intense of the midgut translation product bands.

Conservation of the Jonah Family

If the expression of *Jonah* genes is required for a function essential to the normal development or survival of *D. melanogaster*, we might expect *Jonah* RNA

to be maintained throughout the species. Fourteen wild-type strains, isolated from diverse geographical locations, have been tested for the presence of *Jonah* RNA, and *Jonah* RNA has been detected in all. Figure 9 shows the pattern obtained when third-instar larval RNA from 12 of these strains was assayed by hybridization with the reference cDNA clone, pkdm26H2. Each track contains a single band which comigrates with the band in the Oregon-R track, a band which represents RNA of 0.9 kb in length (Carlson and Hogness, 1985).

The finding that *Jonah* RNA is widely maintained through the *melanogaster* species implies that the existence of at least one *Jonah* gene is maintained; the Oregon-R strain in which we have characterized the *Jonah* genes, however, contains not one but a complex family of approximately 20 *Jonah* genes. The question thus arises as to whether the multiplicity of *Jonah* genes characteristic of Oregon-R is required for normal development or survival. If so, we might expect other wild-type strains of *D. melanogaster* and related flies to contain multiple *Jonah* genes.

Multiple *Jonah* genes are indeed characteristic of other strains of *D. melanogaster*. Figure 10 shows genomic DNA isolated from embryos of Oregon-R, Canton-S, and Hikone strains which has been digested with each of four restriction enzymes, electrophoresed, transferred to nitrocellulose, and hybridized with nick-translated pkdm26H2. At least six bands are visible in all tracks on long exposure. Although the patterns produced by a given enzyme show some common bands, there is also considerable variation among patterns.

Still greater variation in pattern is observed in a similar experiment performed with genomic DNA isolated from other species of *Drosophila* and other flies (M. Akam, unpublished experiments). DNA was extracted from *D. melanogaster* and 3 other species within the same subgroup (the *melanogaster* subgroup: *D. yakuba, D. erecta, D. simulans*), 3 species from different subgroups but of the same subgenus (the *melanogaster* subgenus: *D. willistoni, D. pseudoobscura, D. saltans*), 3 species from a different subgenus (the *Drosophila* subgenus: *D. funebris, D. hydei, D. virilis*), and 3 representatives of other genera (*Musca, Sarcophaga,* and *Calliphora*). The DNA was digested with *Eco*RI, electrophoresed, transferred to ATP paper, and hybridized with aDm3201, a genomic subclone deriving from 99Cβ. Hybridization was detected to the DNA of all species within the *Drosophila* genus, but not to that of the other genera. Among the 10 species of *Drosophila*

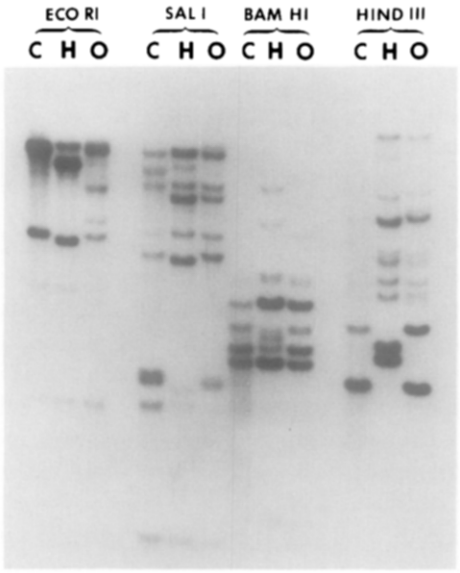

FIG. 10. Hybridization of the *Jonah* reference cDNA clone to genomic DNA of different *Drosophila melanogaster* strains. DNA of three *D. melanogaster* strains was digested with each of 4 restriction endonucleases, electrophoretically fractionated on a 0.5% agarose gel, transferred to nitrocellulose, and hybridized with a ^{32}P-labeled *Jonah* probe (pkdm26H2) as described under Materials and Methods. C, Canton-S; H, Hikone; O, Oregon-R. The Canton-S DNA has suffered some degradation upon incubation with *Bam*HI and *Hin*dIII.

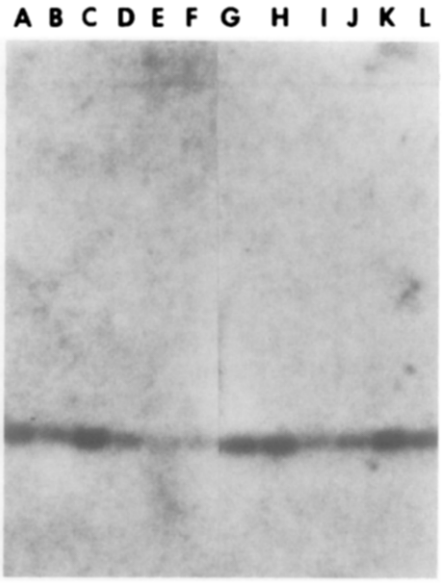

FIG. 9. Hybridization of the *Jonah* reference cDNA clone to RNA of different strains of *Drosophila melanogaster*. RNA was prepared from 50 third-instar larvae of the following strains: A, Urbana-S; B, Swedish-C; C, y^2 *ec cv j car;* D, Chieti-V; E, Oregon-R; F, Seto; G, Hikone-R; H, Canton-S; I, *y w;* J, D323; K, *bw;* L, *cn*. Preparation of filter and hybridization were as described under Materials and Methods. The same results were also obtained with RNA from two other strains: Kochi and BER-1. Fluctuations in levels of hybridization between tracks primarily reflect differences in amounts of RNA per track.

tested, at least four bands are visible in 7 species: *D. virilis, D. willistoni, D. pseudoobscura, D. yakuba, D. erecta, D. simulans,* and *D. melanogaster.* In the cases of the other 3 species, only one to three bands are clearly visible. The number of *Jonah* DNA-containing fragments detectable in this experiment should represent a lower limit to our estimate of the number of such fragments present in these species, as sequence divergence would restrict the number of *Jonah* fragments detectable with the probe under the given hybridization conditions. That the existence of multiple *Jonah* DNA fragments in other strains and species reflects the existence of multiple *Jonah* genes is indicated, in the case of other *D. melanogaster* strains, by *in situ* hybridization experiments, described below, and suggested, in the case of other species, by analogy with the organization of the *Jonah* genes in *D. melanogaster.*

The finding that *Drosophila* of different strains and species contain multiple *Jonah* DNA fragments raises the question of whether *Jonah* genes inhabit a conserved set of chromosomal locations or are variously scattered throughout the chromosomes. Comparisons among species are complicated by the fact that the chromosomal banding patterns of different species are often heterosequential; that is, inversions and other rearrangements accompanying speciation disrupt the pattern of landmarks used to determine chromosomal location. However, comparisons among different wild-type strains of *D. melanogaster* may be made without difficulty, and this approach has been used to demonstrate that in the case of several dispersed repetitive elements, members reside at different sets of sites in different strains. These elements, termed "nomadic" (Young, 1979), are exemplified by the *copia* family (Finnegan *et al.*, 1978). In each of four strains examined, *copia* elements are located at between 19 and 45 sites; however, only one site was common to all four strains (Strobel *et al.*, 1979).

The *Jonah* gene distribution in different strains is very different from that for such mobile elements. *Jonah* genes are present at a minimum of three chromosomal sites, 99C, 99F, and 65A, in all of seven strains tested (Table 2). These strains were of diverse geographical origin and included a Japanese strain, a Swiss strain, and several American strains. These sites were detected using ^{125}I-labeled cDNA clones from *Jon99Cβ* and *Jon65Aiv* as probes. The 99Cβ probe labeled both 99C and 99F in all strains and 65 in three strains. The *Jon65Aiv* probe showed labeling in the 65 region in five strains; the resolution of the chromosomal bands in this experiment did not allow in all cases a precise determination of which lettered subregion of 65 was labeled. A parallel experiment, using a cDNA clone from *Jon25Bi,* allowed identification of a labeled site at 25 in three strains. In Oregon-R, Hikone-B, and Lausanne-S strains, an unexpected site in the vicinity of 62C was tentatively identified; this site had not previously been identified in the *gt 1/gt x-11* chromosomes routinely used in the *in situ* hybridizations described in this and the accompanying paper (Carlson and Hogness, 1985).

DISCUSSION

The *Jonah* gene family is under complex developmental control. Expression is initiated twice, in two different developmental stages. During the larval stage, expression is specific to a single organ, the larval midgut. During the adult stage, expression is confined to the homologous organ, the adult midgut. We have

TABLE 2
SITES OF *IN SITU* HYBRIDIZATION OF FOUR *Jonah* cDNA CLONES TO DIFFERENT STRAINS OF *D. melanogaster*

Strain or genotype	cDNA clone[a]			
	Jon99Cβ	*Jon65Aiv*	*Jon25βi*	*Jon99Cβ*
gt-1/gt x-11	99C (99F)	65A	25B (99C, 99F)	99C, 99F (25B, 44E, 65A, 66C, 67B)
Oregon-R	99C, 99F (65)	65 (62C)	ND	99C, 99F
Canton-S	99C, 99F	65A (99C, 99F)	ND	99C, 99F
Hikone-B	99C, 99F	65A (99C, 99F)	25B, 62C	99C, 99F
Urbana-S	99C (99F, 65A)	ND[b]	ND	ND
Florida	99C, 99F	65	25B	ND
Lausanne-S	99C (99F, 65, 62)	ND	ND	ND

Note. Where letter divisions are indicated, localizations are accurate to within one letter division. In those cases where more than one site was observed, minor sites are listed in parentheses.

[a] Probes were (left to right): adm2374 (*Jon99Cβ*); adm2372 (*Jon65Aiv*); adm2371 (*Jon25Bi*); pkdm26H2 (*Jon99Cβ*).

[b] Not determined.

provided evidence that within this general framework of expression there are differences in the control of different *Jonah* genes, both with respect to developmental time and region of expression. Although we first detect *Jonah* RNA in first-instar larvae, Hafen *et al.* (1983), using the sensitive technique of *in situ* hybridization to RNA in tissue sections, have detected low levels of *Jonah* RNA in presumptive larval midgut cells of 18 hr embryos.

The *Jonah* gene family is complex in its organization as well as in its expression, as described in Carlson and Hogness (1985). The family consists of approximately 20 genes, heterogeneous in structure and distributed in small clusters of up to 4 genes per cluster at eight or more widely dispersed chromosomal sites. What relationships can be drawn between patterns of organization and expression? The property of midgut-specific expression is shared by all *Jonah* genes, or at least by all of those which are expressed as stable transcripts, since we have detected no *Jonah* RNA in any other tissue types. On a finer scale, however, we have shown that different *Jonah* genes vary in their pattern of expression within the larval midgut, and the question thus arises as to whether these differences are cluster-specific: do genes within a cluster show identical patterns of tissue specificity?

Our qualified answer to this question is no, since *Jon65Ai* and *Jon65Aiv* behave differently when hybridized to RNA deriving from different parts of the midgut. The qualification is that this experiment does not assay the levels of *Jon65Ai* or *Jon65Aiv* expression per se, but rather the levels of those RNA species to which these genes bear homology under the given experimental conditions. Thus although *Jon65Ai* is not detectably expressed in the anterior midgut, it is conceivable that *Jon65Aiv* is not expressed in this region either, but bears strong homology to another gene which is.

At the level of gene clusters, expression during the larval stage and expression during the adult stage are not mutually exclusive: our analysis of cDNA clones has shown that genes of the 99Cβ cluster are expressed at both developmental stages. Our experiments suggest that the properties of larval and adult expression are not universal among *Jonah* genes, as the 66C locus is not detectably expressed during the adult stage. We note that the absence of detectable adult expression is characteristic of all genes at this site. Our data on the genetic specificity of *Jonah* expression might conveniently be confirmed and extended using subgenic probes specific for individual *Jonah* RNAs, an approach which has been useful in analyzing the *Dictyostelium* actin multigene family (McKeown and Firtel, 1981).

In vitro translation of hybrid-selected *Jonah* RNA gives rise to a product of 28,000 Da. The *Jonah in vitro* translation product does not comigrate with any of the Coomassie-stained protein bands deriving from midgut extracts. One simple explanation of this result is that the gene product is modified, perhaps by the removal of a signal sequence and/or addition of carbohydrate residues. Alternatively, the products could suffer high rates of turnover or be incorporated into an insoluble matrix. Two-dimensional gel electrophoresis should be useful in discerning whether this band represents a single species or multiple, comigrating species. We might expect *Jonah* translation products to be heterogeneous, since *Jonah* RNA is heterogeneous.

What is the function of the *Jonah* gene products? A large number of enzymes in *Drosophila* have been characterized in terms of molecular weight, tissue distribution, developmental profile, and genetic localization: none of them manifest the characteristics expected of *Jonah* gene products (O'Brien and MacIntyre, 1978). We therefore consider the possibility that the *Jonah* products are structural proteins; specifically, that the *Jonah* genes encode proteins incorporated into a structure known as the peritrophic membrane, a tubular sheath which shields the epithelial cells of the gut from the gut contents (reviewed in Peters, 1976; Wigglesworth, 1965; Kenchington, 1976).

The peritrophic membrane is synthesized continuously in both larvae and adults, and is composed of chitin microfibrils embedded in a matrix consisting mostly of protein and mucopolysaccharide. In third-instar larvae of *D. melanogaster*, the membrane consists of several layers which together are 110–130 nm thick. Adults of other *Drosophila* have been shown to contain two morphologically distinct, concentric peritrophic membranes, PM1 and PM2.

The peritrophic membrane is synthesized at a very high rate: in adult *Calliphora*, another Dipteran fly, peritrophic membranes have been observed to grow at a rate of 3.5 ± 1.0 mm/hr (Zimmerman, 1969, cited in Hepburn, 1976). Moreover, the protein content of the peritrophic membrane has been determined to be 55% in *Calliphora* larvae (Hepburn, 1976), 36% in *Calliphora* adults, and between 21 and 47% in a variety of other insects studied (Wigglesworth, 1965). The high rate of synthesis of peritrophic membrane and its high protein content suggest that a considerable fraction of midgut RNA should encode peritrophic membrane proteins; we have estimated that *Jonah* RNA may constitute as much as 10% of midgut poly(A)$^+$ RNA, and have shown that it gives rise to the most intense band of those produced by *in vitro* translation of midgut RNA.

As mentioned above, the peritrophic membrane is synthesized continuously in larval and adult stages; *Jonah* RNA also is present continuously during these stages. The location of peritrophic membrane protein synthesis has not, unfortunately, been characterized as well as the location of *Jonah* RNA. The peritrophic membrane, or at least a framework thereof, is secreted by cells in the anterior tip of the midgut, close to the region in which *Jonah* RNA has been detected; in addition, one layer of the membrane has been found, in *Calliphora*, to be secreted by delamination of the entire midgut epithelium. There is very little evidence, however, to indicate in which cells peritrophic membrane proteins are synthesized or where within the midgut they are incorporated into the membrane.

It is tempting to speculate that the morphologically different layers of the peritrophic membrane may contain proteins of similar but different structure, perhaps encoded by similar but different genes. Differences in the protein composition of larval and adult peritrophic membranes have been found in *Calliphora* (cited in Hepburn, 1976) as if different peritrophic membrane protein genes are under different temporal control. Direct evidence that *Jonah* genes encode peritrophic membrane proteins might be obtained with antibodies raised against *Jonah* gene products, prepared using any of several expression vectors which have recently become available (e.g., Guarente *et al.*, 1980).

We thank Michael Akam for contributing the results of the hybridization of aDm3201 to the genomic DNA of different species, Mariana Wolfner for her identification of sites in *gt 1/gt x-11* for pkdm26H2 and for her valuable contributions to the work on adm135A8, Marc Muskavitch for donation of the filter containing RNA of different *D. melanogaster* strains, and Tony Mahowald for helpful discussions. This research was supported by grants from the NIH and the NSF. J.C. was supported by an NSF Predoctoral Fellowship and a National Institutes of Health Traineeship.

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