Family of Developmentally Regulated, Maternally Expressed *Drosophila* RNA Species Detected by a *v-myc* Probe

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Drosophila melanogaster genomic sequences that hybridize with *v-myc* have been reported (B.-Z. Shilo and R. A. Weinberg, Proc. Natl. Acad. Sci. U.S.A. 78:6789-6792, 1981). We have detected *Drosophila* RNA sequences that also hybridize with *v-myc*. In an attempt to characterize these RNA sequences, we used *v-myc* hybridization probes to isolate *Drosophila* genomic segments. None of the *Drosophila* genomic or cDNA clones that we have isolated hybridize with the 3' exon of *v-myc*. Preliminary nucleotide sequence analyses have revealed sufficient homology to account for the observed hybridization between *v-myc* and the *Drosophila* clones but have failed to detect significant amino acid sequence homology. Thus it seems unlikely that the mRNA sequences or the genomic sequences that we have isolated by hybridization with *v-myc* represent homologs of the vertebrate *myc* gene. Despite the lack of structural homology between the cloned *Drosophila* sequences and *v-myc*, we have investigated the pattern of expression of those RNA species that hybridize with *v-myc*. Polyadenylic acid-containing transcripts of 2.7, 2.2, and 1.7 kilobases (kb) in embryos, pupae, adults, and Kc cells and an additional 1.4-kb transcript in adults were complementary to the *Drosophila* genomic clones and to *v-myc*. The 1.7- and 2.2-kb transcripts were localized on polyribosomes in Kc cells. The 1.7- and 2.2-kb transcripts were present after 45 min, 2 h, and 4 h of embryonic development, but by 16 h of development their levels had decreased by more than sixfold. During metamorphosis, two peaks of expression of the 1.7- and 2.2-kb transcripts were observed, at 6 and 72 h postpupariation. The 1.4-kb RNA species was first detected at 72 h postpupariation. In adults, the 1.7- and 2.2-kb transcripts were detected only in ovaries in females, whereas the 1.4-kb transcript was present in female nonovarian RNA and in males. These results suggest that the transcripts in early embryos are of maternal origin.

It has been reported that sequences complementary to the *v-myc* oncogene could be detected in the *Drosophila melanogaster* genome by Southern blot hybridization (26). Similarly, we have identified several *Drosophila* RNA species by hybridization with *v-myc*. To characterize these RNA species, we wished to isolate molecular clones containing *Drosophila* genomic sequences. We report here the preliminary characterization of two different *Drosophila* genomic segments isolated by hybridization with *v-myc* and a detailed study of their expression during ontogeny.

The characterization of the *Drosophila* genomic segments isolated by hybridization with *v-myc* reported here indicates that only 5' exon sequences from *v-myc* are complementary to the *Drosophila* sequences. In addition, limited nucleotide sequencing failed to demonstrate amino acid sequence conservation, although significant nucleotide sequence homology was revealed. This is in marked contrast to the results of studies concerning other oncogene-complementary *Drosophila* clones in which nucleotide sequencing studies have shown that each of the three major *v-src*-complementary genomic segments and two of the three *v-ras*-complementary genomic segments are structurally related to the analogous vertebrate oncogene (16, 23; S. C. Wadsworth, K. Madhavan, and D. Bilodeau-Wentworth, manuscript in preparation). Thus it seems unlikely that these *Drosophila* segments represent homologs of the vertebrate *myc* gene. These results bring into question whether the *Drosophila* genome contains *myc*-homologous sequences that can be detected by hybridization techniques.

Despite the lack of structural homology between the *Drosophila* and *v-myc* sequences, the initial results of our investigations into the pattern of expression of the *v-myc*-hybridizing RNA species were novel enough to warrant their completion. Polyadenylic acid-containing transcripts complementary to *v-myc* and to the *Drosophila* genomic segments have been detected in embryos, pupae, adults, and Kc cells and on polyribosomes in Kc cells, suggesting that the *Drosophila* genomic sequences exist in the form of functional genes. We have found that certain transcripts are present in preblastoderm embryos and that their levels fall during midembryogenesis and remain low during the 4 days of larval life. These transcripts reappear during metamorphosis and are present in adults at low levels. In adults, the embryo RNA species are detectable only in ovaries. These results indicate that the early embryonic transcripts are of maternal origin and that their expression may play a role in early development.

MATERIALS AND METHODS

Cell and animal culture. The *Drosophila* Kc₀ cell line (Kc) was maintained on D20 medium (13) (GIBCO Laboratories) containing 5% fetal calf serum (GIBCO Laboratories). Cell lines were maintained in monolayer cultures at 25°C. An Oregon R strain of *D. melanogaster* was used as the source of RNA for all developmental stages analyzed. The sources of the RNA preparations (see Fig. 6) were as follows. Embryos were from an 18-h nonsynchronized collection. Pupae were collected from the walls of culture bottles at 160 h after egg deposition. Synchronization of the pupae was achieved by limiting the time for egg deposition to 2 h. The age of adult flies used for RNA was not controlled.

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For certain experiments (see Fig. 6, 7, and 8), specific developmental stages were collected as follows. For the collection of newly fertilized eggs, well-fed 5- to 6-day-old adults were kept in a food bottle capped with a small agar dish which was smeared with yeast paste and acetic acid for 2 h. After this precollection, eggs were collected for 0.5 h (for 0.75-h-old embryos) or for 1 h (for 2-, 4-, 16-, and 20-h-old embryos) on a fresh, yeast-smeared agar dish. At the end of the collection, the eggs were washed from the agar dishes with distilled water, drained, and stored at -80°C . For the collection of postembryonic stages, well-fed adults were allowed to lay eggs for 1 h in food bottles after a precollection of 2 h. The eggs were allowed to develop, and the different larval instars were collected at the appropriate time, washed in distilled water, and stored at -80°C . Late third-instar larvae crawling on the walls of the food bottle were collected and kept in plastic petri dishes containing wet filter paper. At 0.5-h intervals, white puparia were collected from these dishes and kept in fresh dishes with moist filter paper. These white puparia were staged as 0 h after puparium formation and allowed to develop at 25°C . Animals were taken for RNA extraction at the times described in the text, washed, and stored at -80°C . Soon after their emergence, adult flies were transferred to fresh food bottles and harvested for RNA extraction at the times indicated in the text.

Screening the bacteriophage λ *Drosophila* genome library. The *myc* oncogene segment of the MC29 strain of avian myelocytomatosis virus is contained within a *Pst*I restriction site-terminated DNA fragment of 1,500 base pairs (1), referred to as *v-myc*. The *v-myc* fragment was eluted from preparative agarose gels by electrophoresis and labeled by nick translation (21). A λ phage library of *Drosophila* DNA (20) was screened by hybridization with the *v-myc* probe by the technique of Benton and Davis (4). Specifically, filters were prehybridized for 3 h at 60°C in $4\times$ SET ($1\times$ SET is 0.15 M NaCl, 0.03 M Tris-hydrochloride [pH 8.0], 2 mM EDTA)– $10\times$ Denhardt solution ($1\times$ Denhardt solution is 0.02% each of Ficoll, polyvinylpyrrolidone, and bovine albumin) (12)–0.1% sodium dodecyl sulfate (SDS)–0.1% sodium PP_i and for an additional hour in the same solution containing denatured calf thymus DNA (Sigma Chemical Co.) at 50 $\mu\text{g}/\text{ml}$. After overnight hybridization in the latter solution in the presence of denatured ^{32}P -labeled *v-myc* fragment probe, the filters were washed according to the following schedule: 1 h at 60°C in the hybridization solution, three 45-min intervals at 60°C in $3\times$ SET–0.1% SDS–0.1% sodium PP_i , 45 min at 60°C in $0.3\times$ SET–0.1% sodium PP_i –0.1% SDS, and a final wash in $4\times$ SET at room temperature.

Extraction of RNA. RNA was extracted from cell cultures and from various stages of *Drosophila* development by lysis in 8 M urea–0.1 M Tris-hydrochloride (pH 8.2)–0.3 M NaCl–0.05 M EDTA–4% Sarkosyl. This lysis solution is a modification of the one described by Holmgren et al. (17). CsCl (0.5 g) was added per ml of lysate, which was then layered over a 1.5-ml cushion of 5.7 M CsCl (6). The RNA was pelleted by centrifugation at 36,000 rpm in an SW50.1 rotor for 16 to 18 h. The RNA pellet was dissolved in distilled water containing 0.1% SDS, extracted once with an equal volume of 50% phenol–50% chloroform solution and twice with an equal volume of chloroform, and then ethanol precipitated. Oligodeoxythymidylic acid [oligo(dT)]–cellulose chromatography was performed as suggested by the supplier (Collaborative Research, Inc.). Nuclear and cytoplasmic fractions were prepared by lysing Kc cells in 1% Triton X-100–0.01 M MgCl_2 –0.01 M Tris-hydrochloride (pH

7.6)–0.05 M KCl–0.004 M dithiothreitol–0.25 M sucrose (5). The nuclear pellet was washed once in this solution, and both the cytoplasmic fraction and nuclear pellet were lysed in the 8 M urea solution and pelleted through CsCl as described above. Polyribosomes were prepared by centrifugation of a similar cytoplasmic fraction of Kc cells through sucrose density gradients as described before (10). The gradient fractions corresponding to the polyribosomes, monosomes, and the material sedimenting between monosomes and the top of the gradient were pooled, extracted with phenol plus chloroform, and ethanol precipitated.

RNA and DNA gel electrophoresis, blotting, and hybridizations. Total RNA or oligo(dT)–cellulose fractionated RNA was denatured and electrophoresed in the presence of formaldehyde (19). RNA was denatured at 55°C for 15 min in 50% formamide (99%; Matheson Scientific, Inc.)–6% formaldehyde (Mallinkrodt) with 0.01 M NaH_2PO_4 – Na_2HPO_4 (pH 7.0). The RNA was electrophoresed through horizontal 1% agarose slab gels containing formaldehyde and phosphate buffer as indicated above. The electrode buffer was identical to the gel buffer. Transfer of the RNA to nitrocellulose (Schleicher & Schuell, Inc.) was performed immediately after electrophoresis with $20\times$ SSC ($1\times$ SSC is 0.15 M NaCl and 0.015 M sodium citrate) as the transfer fluid. After overnight transfer, the nitrocellulose sheets were baked at 80°C for 2 h, prehybridized, hybridized, and washed as described previously (29) except that no carrier nucleic acid was used, dextran sulfate was omitted, and 0.5% SDS was incorporated into the hybridization solution. Usually 5×10^6 cpm of ^{32}P -labeled probe prepared by nick translation was hybridized to RNA blots for 12 to 18 h. *Drosophila* gene probes were hybridized at 67°C , and *v-myc* gene probes were hybridized at 60°C .

DNA from the *Drosophila* phage clones or the *v-myc* plasmid was digested with various restriction endonucleases according to the directions of the supplier (Boehringer Mannheim Biochemicals or New England Biolabs). Electrophoresis was through 1% agarose gels except where otherwise indicated. DNA was transferred to nitrocellulose (Schleicher & Schuell, Inc.) with $6\times$ SSC for some experiments (see Fig. 1 and 2) and $20\times$ SSC for another experiment (see Fig. 3) (28). After overnight transfer, filters were baked at 80°C for 2 h. Hybridization probes were prepared by nick translation of gel-purified DNA fragments. The filters were hybridized and washed as described above for hybridization screening of phage libraries. The temperature for hybridization of *Drosophila* gene probes to *Drosophila* sequences was 67°C . Heterologous hybridizations were performed at 60°C .

RESULTS

Isolation of *Drosophila* genomic sequences by hybridization with *v-myc*. *Drosophila* genomic clones were isolated from a λ phage library by hybridization with cloned *v-myc* sequences from avian myelocytomatosis virus (1). Approximately 50,000 plaques (three genome equivalents) were screened on dilute plates. Fifty hybridization-positive plaques were picked and used to prepare small phage DNA preparations. Only those phage which were again *v-myc* positive by Southern transfer hybridization were plaque purified. Eleven phage were eventually obtained. Two of the phage clones, *Dmc6* and *DmcI*, were selected for the studies described in this report. The *Eco*RI digestion patterns and Southern blot hybridization analyses of these clones are shown in Fig. 1A. The *DmcI* clone contained two DNA

FIG. 1. A. Hybridization of the *v-myc* probe to Southern transfers of DNA from *Dmc* phage clones. Two micrograms of DNA from each phage was digested with *Eco*RI, electrophoresed through a 1% agarose gel, and transferred to nitrocellulose. The filters were hybridized with the *v-myc* probe and washed as described in the text. Lanes: a, Ethidium bromide-stained *Eco*RI digest of *Dmc6*; b, autoradiogram of Southern transfer hybridization of a; c, ethidium bromide-stained *Eco*RI digest of *DmcI*; d, autoradiogram of Southern transfer hybridization of c. Approximately 3×10^4 cpm of probe was applied per cm^2 of nitrocellulose. The autoradiographic exposure was 24 h. The size estimates of the *Eco*RI fragments listed in the text were obtained by comparison with a *Hind*III digest of λ DNA run in a parallel lane. B. *Eco*RI restriction maps of *Dmc* genomic clones. The vertical lines indicate the position of *Eco*RI sites within the *Drosophila* inserts. The synthetic *Eco*RI sites connecting the insert to the vector are not shown.

fragments (ca. 7 and 5 kilobases [kb] [Fig. 1A]) that hybridized with *v-myc* are referred to as I-1 and I-2, respectively. Six phage clones were isolated that overlapped the genomic segment in *DmcI* to various extents. Thus the majority of the *Drosophila* sequences that we detected by hybridization with *v-myc* are represented by one genomic locus. The positions of the *Eco*RI sites within these genomic segments are shown in Fig. 1B. The single *Eco*RI fragment within *Dmc6* DNA that hybridized with *v-myc* was ca. 1.5 kb (Fig. 1A). The positions of *Eco*RI sites within *Dmc6* are shown in Fig. 1B. No additional genomic segments overlapping the *Dmc6* clone were isolated.

To compare the cloned DNA to genomic DNA, Southern blot hybridizations were carried out with the gel-purified *v-myc*-hybridizing fragments from the *DmcI* and *Dmc6* clones as probes (Fig. 2). The I-1 and I-2 probes were complementary to genomic *Eco*RI segments indistinguishable in size from the cloned segments. No hybridization to additional genomic sequences was detected. The *Dmc6* probe was complementary to a genomic *Eco*RI segment greater than 23 kb but did not hybridize to the I-1 or I-2 genomic segments. Since the *v-myc*-hybridizing segment in the *Dmc6* clone did not correspond in size to the genomic segment and since the library was established with randomly sheared genomic DNA (20), it was likely that the *Eco*RI site connecting the *Drosophila* sequences to the vector was introduced during cloning. Therefore, to compare the sequences within *Dmc6* to genomic sequences, a sample of the *Eco*RI-cleaved genomic DNA analyzed in lanes B and C (Fig. 2) was further cleaved with *Bam*HI and analyzed by Southern blot hybridization. A genomic *Eco*RI-*Bam*HI fragment that was indistinguishable in size from a cloned *Eco*RI-*Bam*HI segment was detected with the *Dmc6* probe. No hybridization to additional genomic sequences was apparent. On the basis of these results, we concluded that *DmcI* and *Dmc6* represent two different genomic segments.

Hybridization of the *Dmc* clones is restricted to the 5' Exon of *v-myc*. The *myc* sequences in the MC29 virus genome represent most of the amino acid coding sequences present in two exons of the avian *c-myc* gene. It was important to show what portion of the *v-myc* gene was hybridizing with the *Drosophila* clones. The strategy of the following experiment was to digest the *v-myc* DNA fragment with restriction endonucleases that cleave the gene into distinct segments and to perform Southern blot hybridizations with probes specific for the segments of the *Dmc6* and *DmcI* clones that hybridized to *v-myc*. The positions of *Hae*III, *Alu*I, and *Cla*I cleavage sites within *v-myc* are shown in Fig. 3, along with the results of the hybridizations. The *DmcI*-2 probe hybridized with similar intensity to sequences in both the first and second halves of the 5' exon of *v-myc* (Fig. 3a), whereas the *DmcI*-1 probe hybridized with only the second half of the 5' exon of *v-myc* (Fig. 3b). The *Dmc6* probe hybridized strongly to sequences within the second half of the 5' exon of *v-myc* and weakly to the first half of the 5' exon. In a separate experiment, the *Dmc6* homology to the *Hae*III fragment I was mapped to the left of the *Cla*I site in *v-myc* (data not shown).

To test which regions of *v-myc* had the strongest homology with the *Drosophila* probes, the filters from a, b, and c (Fig. 3) were washed at 67°C for two 15-min intervals in $0.3 \times \text{SSC}-0.1\%$ SDS and reexposed. There was an overall reduction in the amount of probe bound, and the *DmcI*-2 fragment hybridization to *Alu*I bands II and III was eliminated (Fig. 3). The hybrids that survived the stringent washing were those from I-2 representing the first half of the 5' exon, those from I-1 representing the second half of the 5' exon, and those from *Dmc6* representing primarily the second half of the 5' exon of *v-myc*.

To better estimate the extent of homology between *v-myc* and the *Drosophila* genomic segments, the *v-myc*-complementary region to the left of the *Eco*RI site in *DmcI* was

FIG. 2. Whole genome DNA Southern blot hybridizations. Two micrograms of DNA isolated from Canton S adults was electrophoresed through a 1% horizontal agarose gel after cleavage with either *Eco*RI (B, C, and D) or *Eco*RI plus *Bam*HI (E). The hybridization probes were prepared from gel-purified, DNA fragments from the λ phage clones *Dmc*I and *Dmc*6 (Fig. 1). Lanes: B, I-2 probe; C, I-1 probe; D and E, 6 probe. In each case, 4×10^5 cpm of probe was applied per cm^2 of nitrocellulose. To mark the positions of the cloned genomic segments, an *Eco*RI digest of *Dmc*I was applied to lane A and was visualized by staining with ethidium bromide. An *Eco*RI-plus-*Bam*HI digest of the 1.5-kb *Eco*RI segment from *Dmc*6 subcloned in m13mp8 was applied to lane F. The positions of the *Drosophila* segments were detected by hybridization with the *Dmc*6 fragment probe. The positions and sizes in kb of *Hind*III-cleaved DNA are shown to the right and left of the panels. The two panels are not aligned at the origin.

subjected to nucleotide sequence analysis (Fig. 3, lower half). This *Eco*RI site splits the *v-myc*-hybridizing region (data not shown). There is 73% sequence homology with *Dmc*I between positions 121 and 163 in *v-myc*. Regions of similar length and percent homology are responsible for the observed hybridization between *v-src* and each of the *Drosophila src* family genes (16; Wadsworth et. al., manuscript in preparation). The overall sequence homology in the region shown is ca. 40%. Despite the demonstrated nucleotide sequence homology, there is no significant amino acid sequence homology. This fact, coupled with the fact that no 3' *v-myc* sequences hybridize to the *Drosophila* clones, suggests that these genomic sequences do not represent true homologs of the vertebrate *myc* gene.

Transcripts detected by *Dmc* clones and *v-myc*. To test whether the *Dmc* DNA sequences were transcribed, RNA was prepared from a variety of sources and chromatographed on oligo(dT)-cellulose. These RNA preparations were electrophoresed through formaldehyde-agarose gels, blotted, and hybridized with probes prepared from fragments isolated from the *Dmc*6 and *Dmc*I clones or the *v-myc* fragment itself (Fig. 4). It can be seen that the two *Drosophila*

ila probes and the *v-myc* probe all hybridized to the same array of RNA species. The *Dmc* probes hybridized more efficiently with *Drosophila* RNA than did the *v-myc* probe. This was evident since 2.5 times the mass of RNA was loaded in panel c and the autoradiographic exposure was 5 times as long as that shown in panels a and b (Fig. 4). This suggested that the cloned *Drosophila* segments were more closely related to these RNA species than is *v-myc*. The three major RNA bands in the Kc lane of panel a are ca. 2.7, 2.2, and 1.7 kb. The intensely hybridizing band seen only in the adult fly RNA is ca. 1.4 kb. This RNA species was always observed in numerous preparations of adult RNA and never observed in the other RNA populations analyzed. The 2.7-kb transcript was most abundant in Kc cell RNA and in adult RNA but barely detectable in pupal and embryo RNA. Smaller transcripts were also detected with these probes but were not consistently observed in different RNA preparations, suggesting that they represented intermediate breakdown products.

The final point to be made from these data is that although the *Dmc*I and *Dmc*6 probes hybridized to the same spectrum of transcripts, the relative intensity of hybridization was characteristic of the probe. The *Dmc*I probe hybridized strongly with the 1.4-kb adult specific RNA and less well with the larger RNAs. The *Dmc*6 fragment probe hybridized efficiently with the 2.7-, 2.2-, and 1.7-kb RNAs but also hybridized with the 1.4-kb transcript. It would therefore appear that some RNA coding sequences were common to both genomic segments but that the *Dmc*6 gene had additional sequences represented in the 2.7-, 2.2-, and 1.7-kb RNAs.

Localization of Kc cell *Dmc*-complementary transcripts on polyribosomes. To further characterize the *Dmc* transcripts present in Kc cells, their intracellular location was determined. Total RNA from whole nucleus and cytoplasm and oligo(dT)-bound RNA isolated from polyribosomes was analyzed by hybridization with the *Dmc*6 probe (Fig. 5). The 2.2- and 1.7-kb transcripts were largely cytoplasmic and were located on polyribosomes. None of the transcripts were concentrated in the nuclear fraction. The hybridization patterns of cytoplasmic and polyribosomal RNA (Fig. 5) differed from the previous results in that the 2.7-kb transcript was low in abundance and there was a continuum of hybridization below the 1.7-kb transcript. It is possible that these RNA preparations suffered more degradation than those analyzed in previous experiments because they were subjected to cell fractionation procedures before the RNA was purified.

Having shown through the above experiments that the 1.7- and 2.2-kb RNA species have some of the properties of mRNA, we wished to further examine their expression during development. In the remainder of the experiments, the *Dmc*6 segment was used as the hybridization probe capable of detecting all *Dmc* RNA species.

Expression of *Dmc* RNA during embryonic development. *Drosophila* embryonic development is completed within 24 h of fertilization (24). Early development is characterized by rapid cycles of chromosome duplication and nuclear division and little, if any, transcription (18, 22, 31). Between 2.5 and 3 h of development, cells are formed around the cleavage nuclei (27), and the first zygotic transcription occurs (18, 22, 31). Total RNA from embryos collected at 45 min (preblastoderm, active nuclear divisions), 2 h (syncytial blastoderm), 4 h (beginning of gastrulation), 16 h (fully formed first-instar larva), and 20 h (embryonic development complete) after egg deposition was hybridized with the *Dmc*6 probe (Fig. 6A).

The predominant transcript detected in 45-min, 2-h, and 4-h embryos was the 1.7-kb RNA; the 2.2-kb RNA was present but was less abundant (Fig. 6A). These transcripts thus were present before the onset of transcription in the embryo. The levels of both transcripts declined between 4 and 16 h of development.

To show that the late embryonic RNA preparations contained intact RNA molecules, a probe representing the amino acid coding region of the *Drosophila* Act5C gene

which cross-reacts with transcripts from other *Drosophila* actin genes (14) was hybridized with embryo RNA (Fig. 6B). Actin transcripts were detected in all stages of embryonic development, including the 16- and 20-h RNA preparations which contained low levels of *Dmc6*-complementary transcripts. In agreement with the published results, the 1.7-kb actin transcript was less abundant in early embryos and more abundant in late embryos relative to the 1.95-kb actin transcript (15).

FIG. 3. Hybridization of *Dmc* fragment probes to digests of the *v-myc* fragment. The positions of cleavage sites for the enzymes *Hae*III and *Alu*I within the *v-myc* fragment are indicated by the vertical dashes above or below the bar diagram in the center of the figure. The position of the single *Cla*I site is indicated below the diagram. The sizes in base pairs of the various DNA fragments denoted by roman numerals are: *Hae*III: I, 470; II, 320; III, 230; *Alu*I: I, 420; II, 320; III, 310; IV, 190. The guanosine-plus-cytosine-rich region referred to in the text is contained within *Alu*I fragment II. The band denoted by P is a partial digestion product of *Alu*I fragments II plus III. The positions of the cleavage sites were taken from the published sequence of *v-myc* (1). The H or A below each lane indicates that the *v-myc* DNA in that lane was digested with either *Hae*III or *Alu*I, respectively. The digests were electrophoresed through 1.5% agarose gels and transferred to nitrocellulose with 20× SSC (28). Each panel, representing adjacent gel lanes, was hybridized with the fragment probe indicated above the individual panels and washed as described in the text except that the 0.3× SET wash was omitted. Panels a, b, and c were from a single gel. The roman numerals adjacent to the various panels denote the positions of the *v-myc* fragments detected in that hybridization. Panels a, b, and c were subsequently washed for two 15-min intervals in 0.3× SSC–0.1% SDS at 67°C and then at room temperature in 2× SSC. The autoradiographic exposures of the rewashed panels were fourfold longer than that shown in panels a, b, and c. Drawn in the lower portion of the figure are the maximum boundaries of hybridization between *v-myc* and the *Dmc* fragments. The nucleotide sequence of the *Dmc*I clone is shown. The I-2 fragment was subcloned into pUC8, cleaved at the *Sal*I site in pUC8, and endlabeled with the large fragment of *Escherichia coli* DNA polymerase I. The method of Maxam and Gilbert was used to determine the sequence of the region to the left of the *Eco*RI site separating the I-2 and I-1 fragments.

As an additional control, the presence of heat shock RNA in the embryo also was investigated. Transcripts from the 83,000 M_r heat shock protein (hsp83) gene have been shown to exist in 1-hour-old embryos and to decline in concentration thereafter (32). When hybridized with an hsp83 gene probe, the embryonic RNA blot (Fig. 6A) revealed the same pattern of expression as that which has been previously demonstrated (Fig. 6C). To quantitate the levels of *Dmc* RNA expression, samples of the same RNA preparations analyzed by gel blot hybridization were dotted onto nitrocellulose and hybridized with the *Dmc6* probe. The intensity of hybridization to the individual dots was normalized to the lowest level observed, that in third-instar larval RNA (Fig. 7). Between 4 and 16 h of embryonic development, the levels of the *Dmc*-complementary transcripts declined by greater than sixfold.

Expression of *Dmc*-complementary RNA during larval development. The larval phase of the *Drosophila* life cycle consists of three instars, the first two being 24 h and the third being 48 h in length. The results of dot hybridizations of the *Dmc6* probe with RNAs from the different larval stages are shown in Fig. 7. The *Dmc* RNA levels which had declined by late embryogenesis remained at similar low levels throughout the three larval instars. The RNA gel blot hybridization pattern of these larval RNA preparations was similar to that observed for the late embryonic RNA preparations. Subsequent hybridization of the larval RNAs with an hsp83 gene probe showed that intact RNA molecules were present in the larval preparations (data not shown).

Expression of *Dmc*-complementary RNA during metamorphosis. The formation of the puparium marks the endpoint of the larval life and the beginning of metamorphosis. During the ensuing 100 h, larval tissues are histolyzed, and the adult is formed as a result of differentiation of imaginal cells. The results of hybridization of the *Dmc6* probe to RNA isolated from metamorphic stages as well as from adults are shown in Fig. 8. The levels of the 1.7- and 2.2-kb RNAs were initially low, increased by 6 h postpupariation, and declined once again before the final peak of expression by 72 h postpupariation. In all metamorphic stages, the 1.7-kb RNA was more abundant than the 2.2-kb RNA. The small RNA molecules that are *Dmc* complementary are believed to be breakdown products of the larger RNA species. The unique feature of the expression pattern at 72 h postpupariation was that it marked the first appearance of the 1.4-kb transcript. The levels of all of the *Dmc* transcripts were rather uniform in adult flies and lower than the 72-h postpupariation sample.

The same RNA blot (Fig. 8A) was subsequently hybridized with the actin 5C probe (Fig. 8B). Actin transcripts could be observed in all lanes, and the overall pattern of hybridization was in agreement with previously published data (15): early-to-middle metamorphic stages contained three sizes of actin transcripts, whereas the predominant transcript during late metamorphosis was the 1.7-kb species.

The relative levels of the *Dmc*-complementary RNAs during metamorphosis were also quantitated by dot hybridizations (Fig. 7). Soon after pupariation, expression transiently increased to levels ca. 10-fold over the third-instar

FIG. 4. Hybridization of *Dmc* and *v-myc* fragment probes to *Drosophila* RNA gel transfers. Oligo(dT)-bound RNA isolated from 20 μ g (or 50 μ g in the *v-myc* panel) of total RNA from embryos (emb.), pupae (pup.), adult flies (ad.), or Kc cells (Kc) was denatured and electrophoresed in the presence of formaldehyde, blotted, and hybridized as described in the text. The hybridization probes indicated at the top of each panel were prepared from the purified *v-myc*-complementary *Eco*RI fragments from the *Dmc* phage clones and from the *v-myc* fragment. Approximately 6×10^4 cpm of probe per cm^2 of nitrocellulose were used. The autoradiographic exposure of panels a and b was 2 days and that of panel c was 10 days. The sizes of the *Dmc* and *v-myc* complementary RNAs were estimated relative to actin mRNAs detected by subsequent rehybridization of the same blots with a *Drosophila* actin probe (Act5C [14]) or relative to heat shock mRNAs in transfers from parallel lanes (9, 17). The size estimates of the *myc*-complementary RNAs are indicated on the right side of the figure. The dashes adjacent to the other panels indicate the positions of these RNAs.

level. The final and largest peak in expression at 72 h postpupariation represented a level ca. 20-fold greater than the third-instar level.

Localization of *Dmc*-complementary transcripts in adults. Since the 2.2- and 1.7-kb transcripts were present in embryos before the onset of transcription, it was probable that the RNAs were of maternal origin. We therefore analyzed RNA from isolated ovaries and from whole adults for the presence of *Dmc*-complementary transcripts (Fig. 9). The levels of the 2.2- and 1.7-kb transcripts in the ovary were equivalent to those observed in whole adult females. The 1.7- and 2.2-kb transcripts were not detected in males or in the remainder of the females from which the ovaries were dissected. Therefore, it is apparent that in adult females, the bulk of the 1.7- and 2.2-kb transcripts was present in the ovary. By taking into account the amount of RNA per female (6 μ g [7]), the fraction that is represented in the ovary (75% [our measurement]), the amount of RNA per oocyte (0.2 μ g [2]), the amounts of RNA loaded per lane, and the relative hybridization levels (embryo level is fivefold greater than ovary level), we concluded that the majority of the 2.2- and 1.7-kb transcripts observed in ovary RNA is destined for deposition in the oocyte.

DISCUSSION

The principal goal of the experiments described in this report was to characterize the *Drosophila* RNA sequences

FIG. 5. Hybridization of the *Dmc6* fragment probe to RNA from fractionated Kc cells. Five micrograms of total RNA from cytoplasm (Cyto.) and nuclear RNA representing the same number of cells (Nuc.) or the oligo(dT)-bound fraction of ten micrograms of RNA from polyribosomes (Polys.) was denatured and electrophoresed in the presence of formaldehyde, blotted, and hybridized as described in the text. The hybridization probe was prepared from the *v-myc*-complementary *Eco*RI fragment from the *Dmc6* phage clone.

FIG. 6. RNA gel transfer hybridization analysis of embryonic development. Two micrograms of total RNA from the stages indicated at the top of the lanes was electrophoresed, transferred to nitrocellulose, and hybridized as described in the text. The hybridization probe in A was the *Dmc6* fragment. The numbers on the left indicate the positions of the *Dmc* transcripts and their sizes in kb. In B and C, the hybridization probes were a *Drosophila* actin gene clone (Act5C [14]) and the hsp83 gene clone (32), respectively. Only the portions of the autoradiograms that correspond to actin and hsp83 mRNAs are shown in B and C. The same nitrocellulose filter used for A was later used for C.

detected by hybridization with the vertebrate *myc* gene. To facilitate these analyses, we isolated two *Drosophila* genomic segments by hybridization with *v-myc*. As noted in the discussion, the available evidence indicates that these *Drosophila* genomic segments do not represent homologs of the vertebrate *myc* gene. Nucleotide sequencing of the *DmcI* genomic clone revealed sufficient homology to account for the observed hybridization, but no significant amino acid sequence homology was apparent. More discussion on this point follows below.

By using the *Dmc* clones as hybridization probes, we were able to achieve the original goal of characterizing the *Drosophila* RNA species that hybridized with *v-myc*. The levels of the 1.7- and 2.2-kb RNAs are constant at least through the beginning of gastrulation but decrease in abundance between 4 and 16 h of embryonic development. Because the preblastoderm embryo is not transcriptionally active (18, 22, 31), it is probable that the early embryonic transcripts are of maternal origin. This hypothesis is strengthened by our demonstration that the preblastoderm embryo RNA species are found predominantly in ovaries of adult females. During the late embryonic and larval stages, the levels of the *Dmc*-complementary transcripts were consistently low but detectable. Since the embryonic transcripts would have been diluted during larval growth, the presence of *Dmc*-complementary RNAs must be due to continued transcription during the larval stages.

FIG. 7. Dot hybridization analysis of RNA from different stages of *Drosophila* development. RNA from the stages indicated was boiled in water and quenched on ice, and 1 μ g of RNA from each stage was dotted onto nitrocellulose (29). The strip of nitrocellulose containing all of the RNA dots was hybridized with the *Dmc6* fragment probe as described in the text. The resulting autoradiogram was scanned, and the relative hybridization was normalized to the lowest intensity measured, that representing third-instar larval RNA. The RNA preparations used for dot hybridization were the same preparations analyzed by RNA gel transfer hybridizations. The values shown are the average of three determinations

Metamorphosis, like embryonic development, is characterized by a rapid pace of differentiation. Two peaks of expression of the 1.7- and 2.2-kb transcripts, as well as the first appearance of the 1.4 kb transcript, were documented during metamorphosis. We presently cannot determine the locations of the *Dmc*-complementary RNAs in embryonic, larval, and metamorphic stages. However, the fact that

expression of the 1.7- and 2.2-kb RNA species was observed only in ovaries in adults suggests that the transcripts observed in earlier developmental stages also might be restricted to cells that give rise to ovaries. It is apparent from our results that the levels of *Dmc*-complementary RNA species are highest in developmental stages characterized by intense differentiation and cell division and lowest in the

FIG. 8. RNA gel transfer hybridization analysis of metamorphic and adult stages. Two micrograms of total RNA from the stages indicated at the top of the lanes was electrophoresed, transferred to nitrocellulose, and hybridized as described in the text. The hybridization probe in A was the *Dmc6* fragment. The numbers on the left and right indicate the positions of the *Dmc* transcripts and their sizes in kb. The hybridization probe in B was the *Drosophila* actin gene clone. Only the portion of the autoradiogram corresponding to actin mRNA is shown in B.

FIG. 9. Hybridization of RNA from embryos, ovaries, and carcass (from which ovaries were dissected) females and males. Ovaries were hand isolated from 5-day-old, well-fed females. RNA was extracted from the ovaries as well as from the remainder of the animal (carcass). RNA was prepared from total animals of both sexes from the same population of adults. The embryo RNA is a sample of the same RNA preparation from 0.75-h embryos analyzed in Fig. 6. Two micrograms of RNA was applied per lane.

larval stages. The extent of the evidence bearing upon the form of the *Dmc*-complementary RNAs is that they all contain polyadenylic acid and that the 1.7- and 2.2-kb species are localized on polyribosomes in the Kc line of cultured *Drosophila* cells. Thus if the *Dmc*-complementary RNA species do encode proteins, the observed pattern of expression suggests an involvement of such proteins in early embryonic development.

The data presented do not permit an assignment of specific transcripts either to the *Dmc6* or *DmcI* clones or to others that we have isolated (see below). However, it is important to note that the *Dmc6* and *DmcI* genomic sequences did not detect additional RNA species that were not also detected by *v-myc*. Thus, it is apparent that the observed RNA species are transcribed from members of a *Drosophila* gene family exemplified by the *Dmc6* and *DmcI* clones. To address the question of which genomic segments represent the genes encoding the individual transcripts, we have recently isolated cDNA clones representing the embryonic RNAs (S. Wadsworth, unpublished data). A detailed comparison of the genomic and cDNA sequences is in progress.

Evidence has been presented that indicates that the *Dmc6* and *DmcI* clones represent two different genomic segments that hybridize with *v-myc*. The Southern blot hybridization results of Shilo and Weinberg (26) indicated that the sequences in the *Drosophila* genome that hybridized with *v-myc* were contained in several restriction endonuclease-generated DNA fragments. Taken together, these results suggest that the sequences within the *Drosophila* genome detected by *v-myc* are organized as a gene family. Relevant to this hypothesis, we isolated two additional genomic segments in parallel with *Dmc6* and *DmcI*. The two additional clones have characteristics common to *Dmc6* and *DmcI*; they hybridize to sequences only within the 5' exon of *v-myc*, they share *v-myc*-hybridizing regions with each other and the *Dmc6* and *DmcI* clones, and they are complementary to the same set of RNA species seen in Fig. 6. Therefore, the bulk of the *Drosophila* genomic sequences that can be detected by *v-myc* hybridize exclusively with the 5' exon of *v-myc*. In addition, we have isolated cDNA clones representing the 2.7-, 2.2-, and 1.7-kb RNA species which, like the genomic clones, are not complementary to the 3' exon of *v-myc*. Therefore, the existing data suggest that the

Drosophila genomic and RNA sequences that we have identified by hybridization with *v-myc* represent genes structurally distinct from the vertebrate *myc* gene. We have screened the same genomic library without success with hybridization probes specific for the 3' exon of *v-myc* (the *Cla*I-to-*Pst*I fragment [1]). Whether the *Drosophila* genome contains sequences analogous to the 3' exon of *v-myc* that can be detected by hybridization techniques remains to be resolved.

The existence of several different *Drosophila* genomic sequences that hybridize with *v-myc* is reminiscent of recent observations on the arrangement of *myc*-hybridizing sequences in the human genome. One *c-myc* gene homologous to both *v-myc* exons has been identified (3, 8, 30), whereas four additional genomic segments hybridizing to the 5' exon of *v-myc* have been identified (11, 25). The homology between *v-myc* and one such *myc*-related gene, *N-myc*, has been shown to be due to two, short, discontinuous blocks of conserved sequence (25). Because of the interesting pattern of expression that we have demonstrated here for the *Dmc* sequences, we are undertaking a detailed structural analysis of the genomic clones to definitively assess their relationship to the vertebrate *myc* gene or other vertebrate genes.

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