Isolation of hnRNP Complexes from *Drosophila melanogaster*

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Abstract. Nascent RNA polymerase II transcripts, heterogeneous nuclear RNAs (hnRNAs), become associated with nuclear proteins (hnRNP Proteins), and their processing into mRNAs takes place in these hnRNP complexes. hnRNP complexes have previously been purified from vertebrate cells. Here we report the isolation of hnRNP complexes from an invertebrate organism, the fruitfly *Drosophila melanogaster*. Candidate hnRNP proteins were purified from *D. melanogaster* embryos by ssDNA affinity chromatography, and mAbs were produced to many of the major proteins. Genuine hnRNP proteins were identified by several criteria, including nucleoplasmic localization, association with nascent transcripts, crosslinking to poly(A)-containing RNA in living cells, and amino acid sequence. In addition, mAbs that cross-react between the fruitfly and human hnRNP proteins were obtained. Most importantly, using hnRNP-specific mAbs we have purified the hnRNP complexes from *D. melanogaster* cells. These RNAase-sensitive complexes contain at least 10 major proteins designated hrps, the most abundant proteins having apparent molecular masses of 36, 38, 39, 40, 44, 48, 54, 62, 70, and 75 kD. cDNAs and complete sequences for several of these proteins have been obtained and are presented in the accompanying paper (Matunis, E. L., M. J. Matunis, and G. Dreyfuss. 1992. *J. Cell Biol.* 116:257-269). The purification of *D. melanogaster* hnRNP complexes will facilitate genetic and cytological studies on the function of hnRNA-binding proteins and on the posttranscriptional regulation of gene expression.

IN eukaryotic cells, RNA polymerase II transcripts undergo a series of processing events required to convert them from heterogeneous nuclear RNAs (hnRNAs)[1] into mature mRNAs. These processing events, which include capping, polyadenylation, splicing, and nucleocytoplasmic transport, constitute an important part of the pathway of gene expression (Darnell, 1982). Although details of the molecular mechanisms required for converting hnRNA to mRNA remain largely unknown, these processing reactions appear to occur in RNA-protein complexes which form as the hnRNA is being transcribed (Gall and Callan, 1962; Malcolm and Sommerville, 1974; Beyer and Osheim, 1988; Wu et al., 1991). In general, two classes of components interact with hnRNAs: heterogeneous nuclear ribonucleoproteins (hnRNPs) and small nuclear ribonucleoprotein (snRNP) complexes. Although snRNP complexes are known to be intimately involved in pre-mRNA splicing (for reviews see Guthrie and Patterson, 1988; Zieve and Sauterer, 1990) relatively little is known about the precise functions of the hnRNP proteins.

hnRNP proteins form stable complexes with the hnRNA and are associated with it until its export from the nucleus as mRNA (for reviews see Chung and Wooley, 1986; Dreyfuss, 1986; Dreyfuss et al., 1988). Much of the current knowledge about the structure and composition of hnRNP complexes has come from studies of vertebrate cells, particularly human HeLa cells (Pederson, 1974; Beyer et al., 1977; Karn et al., 1977; Brunel and LeLay, 1979; Choi and Dreyfuss, 1984*a*; Leser et al., 1984; Wilk et al., 1985; Piñol Roma et al., 1988). hnRNP complexes immunopurified with specific mAbs are composed of over 20 abundant nuclear proteins designated A1 through U (Choi and Dreyfuss, 1984*a*; Piñol Roma et al., 1988; Piñol Roma et al., 1989). Complexes immunopurified from human, hamster, and chicken cells have a similar protein composition, indicating that many of the hnRNP proteins are conserved in vertebrates (Choi and Dreyfuss, 1984*a*; Matunis M., S. Piñol Roma, and G. Dreyfuss, manuscript submitted for publication). In addition, hnRNP complexes of a similar general structure have been detected by sucrose gradient sedimentation from a wide range of organisms, including invertebrates (Firtel and Pederson, 1975; Beyer et al., 1977; Brunel and Lelay, 1979; Thomas et al., 1981; Risau et al., 1983). However, even though individual candidate hnRNP proteins have been identified in *Drosophila melanogaster, Artemia salina*, and *Physarum polycephalum* (Christensen et al., 1977; Thomas et al., 1981; Risau et al., 1983; Haynes et al., 1987, 1990, 1991), invertebrate hnRNP complexes have not been purified or analyzed in detail.

Invertebrate organisms, particularly *D. melanogaster*, offer several important advantages for investigating the role of hnRNP proteins in pre-mRNA metabolism. In addition

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1. *Abbreviations used in this paper:* hn, heterogeneous nuclear; sn, small nuclear; ss, single stranded.

to providing a well characterized genetic system in which to study protein function, the polytene chromosomes of *D. melanogaster* allow for direct observation, by immunological methods, of proteins associated with nascent RNA transcripts (Silver and Elgin, 1977). In this study, we have utilized the single-stranded nucleic acid–binding properties of hnRNP proteins (Pullman and Martin, 1983; Wilk et al., 1983; Pandolfo et al., 1987; Piñol Roma et al., 1988) to purify them from *D. melanogaster* embryos. mAbs were produced to many of these proteins and used to characterize the components of *D. melanogaster* hnRNP complexes. Antibodies specific for one of the most abundant hnRNP proteins, hrp40, have allowed us to immunopurify hnRNP complexes from *D. melanogaster*. In addition, cDNAs and sequences for several of the most abundant hnRNP proteins have been obtained and are presented in the accompanying paper (Matunis et al., 1992). Together, these data provide a detailed map of *D. melanogaster* hnRNA-binding proteins and a reference for the characterization of additional hnRNP processing components.

Materials and Methods

Cell Culture and Labeling

Schneider's line 2 *D. melanogaster* cells (S2) were cultured at 25°C in modified Schneider's *Drosophila* medium (Gibco BRL Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% FCS, penicillin, and streptomycin. Cells were labeled in the same media containing one-tenth the normal concentration of methionine, one-fifth the normal concentration of yeastolate (to further reduce the concentration of free methionine and enhance labeling efficiency), 5% FCS, and 20 μCi/ml [^{35}S]methionine.

Cell Fractionation

All steps of the fractionation were carried out at 4°C. Frozen, dechorionated, Oregon-R embryos (2–18 h) were generously provided by Dr. Anthony Mahowald of Case Western Reserve (presently at the University of Chicago). Generally, 10 ml of frozen embryos were thawed directly into 20 ml of RSB-100 (10 mM Tris-HCl [pH 7.4], 100 mM NaCl, 2.5 mM MgCl$_2$) containing 0.5% Triton X-100, 0.5% Aprotinin, 2 mg/ml leupeptin, and 2 mg/ml pepstatin A (Sigma Chemical Co., St. Louis, MO). Embryos were lysed by sonication, 4× 30 s bursts with one minute of cooling between each burst. Lysates were centrifuged at 1,000 g for 10 min to remove particulate matter. The supernatant was layered over a 30% sucrose cushion (30% sucrose in RSB-100) and centrifuged for 15 min at 5,000 g to further remove chromatin, nucleoli, and other particulate material without sedimenting RNP complexes. The top layer was collected and used for further fractionation.

Cultured S2 cells were pelleted, washed once in PBS, and resuspended in the same buffer used to lyse embryos (described above). Cells were sonicated 3× for 5 s with 30 s of cooling between each burst. The lysates were then fractionated on a 30% sucrose cushion as described above. The top layer was collected and used for further fractionation. Empigen BB lysate was prepared by resuspending cells in PBS containing 1% Empigen BB (Albright and Wilson Inc., Norwood, NJ), 0.1 mM DTT, 1 mM EDTA, and protease inhibitors (0.5% Aprotinin, 2 mg/ml leupeptin, and 2 mg/ml pepstatin A). Cells were sonicated as described above and centrifuged at 16,000 g for 5 min. The supernatant was used directly for immunopurification.

RNase Digestion

Cell lysates were digested with micrococcal nuclease (Pharmacia LKB Biotechnology, Piscataway, NJ) at 100 U/ml for 10 min at 30°C in the presence of 1 mM CaCl$_2$. Reactions were stopped on ice by the addition of EGTA to a final concentration of 5 mM.

ssDNA Chromatography

Proteins were fractionated by ssDNA affinity chromatography essentially

as described (Piñol Roma et al., 1988, 1990). After micrococcal nuclease digestion, proteins were bound to ssDNA-cellulose (United States Biochemical Corp., Cleveland, Ohio) at 0.1 M NaCl. Extracts prepared from embryos were bound to a 10-m column, and a smaller, 1-ml column was used for experiments involving two to four flasks of cultured, labeled cells. Columns were washed with 1 mg/ml heparin in 0.1 M NaCl and eluted with the designated NaCl concentrations. HeLa cell ssDNA-binding proteins were isolated as previously described (Piñol Roma et al., 1988, 1990).

Preparation of mAbs

mAbs were prepared by immunizing BALB/c mice with ssDNA-binding proteins purified from Oregon-R embryos. Hybridoma production and screening was performed essentially as previously described (Choi and Dreyfuss, 1984a; Dreyfuss et al., 1984a) except that hybridomas were screened by immunofluorescence on S2 cells. Antibody specificities were determined by two-dimensional immunoblots and by immunoprecipitation in the presence of the ionic detergent Empigen BB (Choi and Dreyfuss, 1984a).

Gel Electrophoresis and Immunoblotting

Proteins were separated by SDS-PAGE and fluorographed (where appropriate) as previously described (Dreyfuss et al., 1984a). For Coomassie blue detection, protein gels were first transferred to immobilon-P membrane (Millipore Corp., Bedford, MA) and the membranes were then stained with Coomassie blue. Two-dimensional NEPHGE was performed as described by O'Farrell et al. (1977) using an ampholine gradient of pH 3–10 separated for 4 h at 400 V in the first dimension. Proteins were separated by SDS-PAGE in the second dimension. Immunoblotting was carried out as previously described (Choi and Dreyfuss, 1984b). Proteins were transferred to nitrocellulose membrane (Schleicher & Schuell, Keene, NH) and probed with the indicated mAbs. Generally, all mAbs were used in the form of ascites fluid diluted 1:1,000, except antibody 3A, which was mouse polyclonal serum used at a similar dilution.

Immunopurification of hnRNP Complexes

hnRNP complexes were immunopurified from *D. melanogaster* postchromatin-nucleolar whole cell extracts in the presence of 0.5% Triton X-100 (prepared as described above) essentially as previously described (Choi and Dreyfuss, 1984a). Extracts were incubated with the monoclonal antibody 8G6 (prebound to protein-A agarose [Pharmacia LKB Biotechnology, Piscataway, NJ]) for 15 min at 4°C. The protein-A agarose was spun down, washed 4× with RSB-100 containing 0.5% Triton X-100 and protease inhibitors (0.5% Aprotinin, 2 mg/ml leupeptin, and 2 mg/ml pepstatin A) and the beads were resuspended directly in SDS-PAGE sample buffer. Antibody specificities were determined by similar immunopurifications from whole cell lysates, but in the presence of the ionic detergent Empigen BB (prepared as described above). Ascites fluid from a BALB/c mouse inoculated with the parent myeloma cell line SP2/0 was used in each experiment as a nonimmune control.

RNA-Protein Cross-linking in Intact Cells

Photochemical RNA-protein cross-linking by UV-light irradiation of cells and isolation and analysis of RNPs was carried out essentially as previously described for human HeLa cells (Dreyfuss et al., 1984a). S2 cells were pelleted and resuspended in PBS at ~1 × 10^6 cells/ml. 3 ml of this cell suspension was placed in a cell culture dish and the cells were irradiated for 3 min. Cells were lysed as previously described except that nuclei and cytoplasm were not separated. Poly(A)-containing RNA was isolated by chromatography on oligo(dT)-cellulose (Collaborative Research Inc., Bedford, MA) and bound proteins were analyzed as previously described (Dreyfuss et al., 1984a).

Immunofluorescence Microscopy

S2 cells were attached to slides by centrifuging at 200 rpm for 30 s in a Cytospin 3 centrifuge (Shandon Inc., Pittsburgh, PA). Cells were fixed in PBS containing 2% formaldehyde for 30 min and permeabilized by incubation for 3 min in acetone at −20°C. Immunofluorescence microscopy was carried out as previously described (Dreyfuss et al., 1984a) using a Zeiss Axiophot photomicroscope. Detection was with FITC-conjugated goat antimouse F(ab')2 (Cappel Laboratories, Malvern, PA). All antibodies were used in the form of ascites fluid (except 3A, which was a mouse polyclonal serum) at a dilution of 1:1,000.
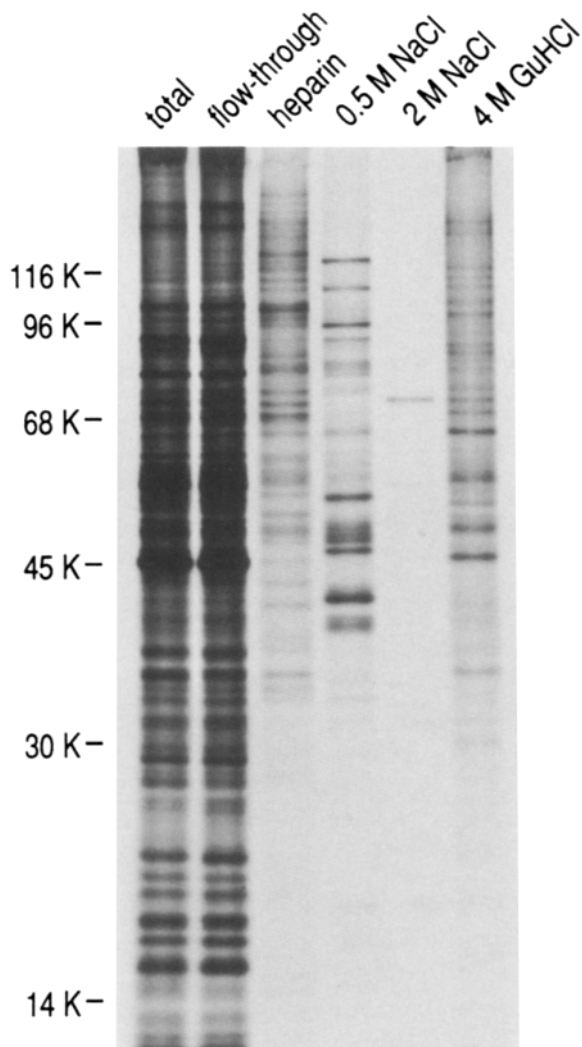
Figure 1. Purification of candidate RNP proteins by ssDNA chromatography. [^{35}S]methionine-labeled S2 cell lysate was bound to ssDNA-cellulose at 0.1 M NaCl. The column was washed with 1 mg/ml heparin and eluted with 0.5 M NaCl, 2 M NaCl, and 4 M guanidine HCl (GuHCl). Proteins in each fraction were TCA precipitated, resolved by SDS-PAGE, and detected by fluorography. Molecular weights of the protein standards are indicated.
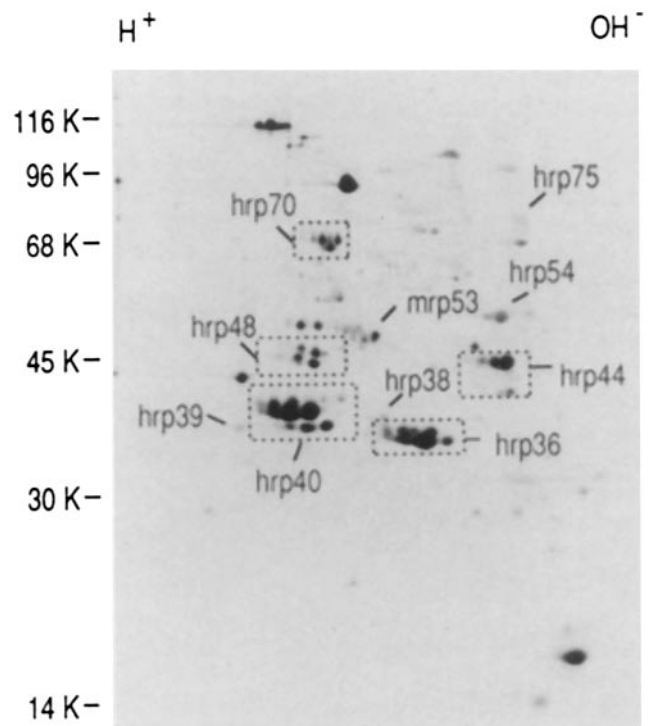
Figure 2. Two-dimensional gel electrophoresis of proteins purified by ssDNA chromatography. S2 cell lysate was bound to ssDNA-cellulose at 0.1 M NaCl. The column was washed with 1 mg/ml heparin and proteins were eluted with 2 M NaCl. Proteins were resolved by two-dimensional gel electrophoresis (NEPHGE in the first dimension, SDS-PAGE in the second dimension), transferred to immobilon-P membrane, and stained with Coomassie blue. Nomenclature for the indicated proteins is described in the text. Molecular weights of the protein standards are indicated.

Polytene Chromosome Immunofluorescence

Salivary glands from third instar Oregon-R larvae were dissected in Cohen and Gotchell medium G with 0.5% NP-40, fixed in formaldehyde fixative, and squashed in 45% acetic acid as described in Ashburner (1989). Squashes were stained with a 1:1,000 dilution of each mAb, and detected with a 1:50 dilution of FITC-conjugated goat antimouse F(ab')2 (Cappel Laboratories, Malvern, PA).

Results

Isolation of Candidate hnRNP Proteins from Drosophila melanogaster

hnRNP proteins have been purified from various vertebrate cell lines by affinity chromatography on immobilized ssDNA (Pandolfo et al., 1987; Piñol Roma et al., 1988; Matunis M., S. Piñol Roma, and G. Dreyfuss, manuscript submitted for publication). To identify candidate hnRNP proteins from *D. melanogaster*, [^{35}S]methionine-labeled proteins from Schneider's line 2 (S2) whole cell extracts were fractionated

by chromatography on ssDNA-cellulose (Fig. 1). Many proteins which remain bound to the column after a heparin wash elute with 0.5 M NaCl, and a major protein of 70 kD elutes with 2 M NaCl. Of particular interest are the abundant proteins that elute with 0.5 M NaCl and are in the molecular mass range of 30–68 kD, as the major vertebrate hnRNP proteins are of this relative size and have similar ssDNA-binding characteristics (Pandolfo et al., 1987; Piñol Roma et al., 1988). To further resolve the purified proteins, the 0.5 and 2 M NaCl ssDNA fractions were combined and analyzed by two-dimensional gel electrophoresis (Fig. 2). A comparison with two-dimensional gels of HeLa ssDNA-binding proteins (see Piñol Roma et al., 1988) reveals some common general features. For example, the major proteins are in the molecular mass range of 30–48 kD and many additional proteins are apparent, suggesting that *D. melanogaster* also has a large assortment of hnRNP proteins. In addition, several of the most abundant ssDNA-binding proteins are resolved into clusters of spots, probably representing related protein isoforms. In particular, three groups of acidic proteins of 40, 48, and 70 kD, and two groups of more basic proteins of 36 and 44 kD are resolved. There are also several abundant, higher molecular mass proteins of ∼90 and 100 kD, and a small basic protein of 20 kD. Although the identity of the 20-kD protein is not known, it is noteworthy that UP1, a 24-kD protein often observed in ssDNA chromatography of mammalian cells, has been demonstrated to be a proteolytic fragment of the A1 hnRNP protein (Pandolfo et al., 1985). Using the cri-

teria presented below many of the *D. melanogaster* ssDNA-binding proteins are genuine hnRNP proteins. However, the identification of hnRNP protein homologues between *D. melanogaster* and human is not possible based on a comparison of two-dimensional gels. We have therefore designated the isolated proteins according to their relative size preceded by "hrp" or "mrp" to distinguish between heterogeneous nuclear RNP and messenger RNP. Many of the proteins have also been grouped, as they appear to be isoforms by antibody reactivity and nucleotide sequence (see below and Matunis et al., 1992). Previous designations for *D. melanogaster* hnRNP proteins (e.g., *HrbX*; X = genetic locus; Haynes et al., 1990, 1991) were not suitable because the genes for different hnRNP proteins colocalize (Matunis et al., 1992) and because localizations are not known for all the identified proteins.

mAbs to D. melanogaster hnRNP Proteins

To obtain antibodies specific for individual hnRNP proteins, we immunized mice with ssDNA-binding proteins purified from *D. melanogaster* embryos (from which large quantities of protein can be obtained) as described in Materials and Methods. Overall, the proteins isolated from embryos were similar to those isolated from S2 cells as determined by two-dimensional gel electrophoresis (data not shown). Fig. 3 shows an immunoblot of a ssDNA purification from S2 cells probed with several of the mAbs that were obtained. Antibodies 4A7, 10D5, 8G6, and 5A5 recognize proteins of 53, 48, 40, and 36 kD, respectively, which elute from ssDNA at 0.5 M NaCl. mAb 5C11 recognizes the major 70-kD protein eluting with 2 M NaCl. To ensure complete elution of all the bound proteins, the column was also washed with 4 M guanidine HCl. Although many of the identified proteins elute with 4 M guanidine HCl, this likely represents solubilization of proteins which have aggregated on the column, rather than elution of a subset of proteins with higher affinity for ssDNA (see Piñol Roma et al., 1988). The antigen recognized by 6E2 (65 kD) has been characterized as the cytoplasmic poly(A)–binding protein (PABP) and is present primarily in the flow-through and the heparin wash, consistent with properties of the yeast PABP (Matunis, M., and G. Dreyfuss, unpublished results). To more clearly define the proteins recognized by the antibodies, two-dimensional protein blots of total S2 lysate were probed (Fig. 4). Many of the mAbs recognize several protein spots, further suggesting that the clusters evident in Fig. 2 are related isoforms, possibly generated by posttranslational modifications or by alternative pre-mRNA splicing. A list of the antibodies discussed, and the proteins they recognize, is presented in Table I. Antibodies 8G6, 8D2 (two-dimensional immunoblots not shown), 4C2, 3C8, and 6C7 all react with a group of approximately eight acidic proteins in the molecular mass range of 38–40 kD, designated hrp40. The mAb 4A7 recognizes mrp53; 5A5 recognizes at least three proteins which have been designated hrp36; 10D5 recognizes the six polypeptides of hrp48; 5C11 recognizes hrp70; and 3A recognizes the hrp44 cluster. *C* and *E* were probed sequentially with the designated mAbs.

The cellular localizations of the proteins identified by immunoblotting were determined by indirect immunofluorescent staining of S2 cells. Fig. 5 shows the subcellular localization of the proteins recognized by 4C2, 5A5, 3A, 5C11, and 4A7. hrp40 (*A* and *B*), hrp44 (*G* and *H*), and hrp70 (*E* and *F*) are located in the nucleus, excluding nucleoli. Al-
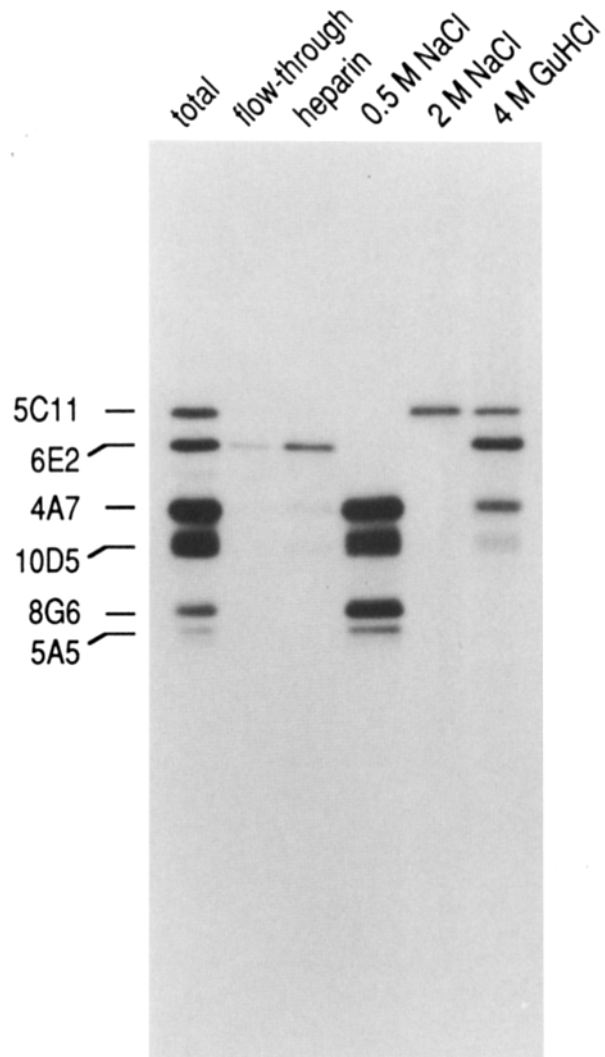

Figure 3. Immunoblot analysis of antibodies to the major ssDNA-binding proteins. Protein fractions similar to those in Fig. 1 were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and probed simultaneously with six different mAbs: 5C11 (anti-hrp70), 6E2 (anti-PABP), 4A7 (anti-mrp53), 10D5 (anti-hrp48), 8G6 (anti-hrp40), and 5A5 (anti-hrp36). Antibodies were detected with ^{125}I-labeled goat antimouse.

though a low level of cytoplasmic staining is consistently observed with the antibodies specific for these proteins, as compared to hrp36, which appears exclusively nuclear (*C* and *D*), we do not know whether this represents actual cytoplasmic localization of these proteins or nonspecific background staining. *I* and *J* illustrate the localization of mrp53 which is exclusively cytoplasmic. Because of its cellular localization and its affinity for single-stranded nucleic acids, mrp53 is a putative cytoplasmic mRNA-binding protein.

In addition to their nuclear localization, the proteins we have examined are also associated with actively transcribing loci of polytene chromosomes. The puffing of loci is in general a direct reflection of transcriptional activity and, therefore, of the presence of high concentrations of nascent hnRNAs (Jamrich et al., 1977; Silver and Elgin, 1977; Kabisch and Bautz, 1983). The localization of hnRNP proteins to these regions of the polytene chromosomes represents binding to nascent RNA transcripts. With antibodies to hrp36, hrp40, and hrp48, intense staining is seen at the active
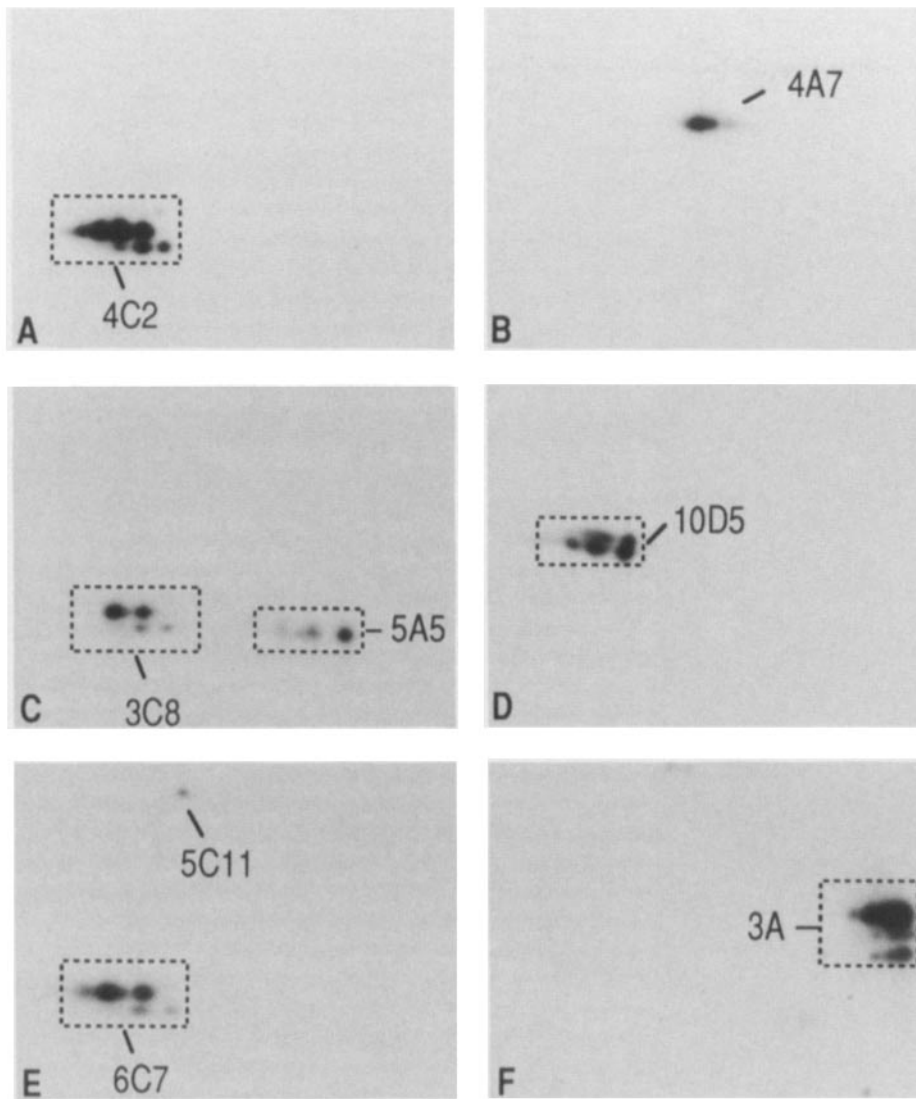
Figure 4. Two-dimensional immunoblot analysis of antibodies to the major ssDNA-binding proteins. Total S2 cell proteins were resolved by two-dimensional gel electrophoresis, transferred to nitrocellulose membrane, and probed with the indicated antibodies: *A,* 4C2 (anti-hrp40); *B,* 4A7 (anti-mrp53); *C,* 3C8 (anti-hrp40) and 5A5 (anti-hrp36); *D,* 10D5 (anti-hrp48); *E,* 5C11 (anti-hrp70) and 6C7 (anti-hrp40); *F,* 3A (anti-hrp44). Blots probed with more than one antibody were probed sequentially and exposed to film after the binding of each antibody.

loci (puffs) and less intense, or no staining, is observed in the interbands (Fig. 6). At the level of resolution used in this study, all of the actively transcribing loci appear to be stained equally with the different mAbs, in a pattern similar to that

Table I. D. melanogaster RNP Proteins and Antibodies

Protein*	Antibody‡
hrp36	5A5
hrp38	N/A
hrp39	N/A
hrp40	4C2, 8G6, 3C8, 6C7, 8D2
hrp44	3A§
hrp48	10D5
mrp53	4A7
hrp54	N/A
hrp62	N/A
hrp70	5C11
hrp75	N/A
PABP	6E2

* hrp, heterogeneous nuclear ribonucleoprotein; mrp, messenger ribonucleoprotein; PABP, Poly(A)-binding protein. Number following hrp or mrp designates apparent molecular weight.
‡ N/A, antibody not available.
§ Indicates mouse polyclonal serum.

seen with anti-RNA polymerase B antibodies (Jamrich et al., 1977).

The hrp40 proteins, which appear to be the most abundant single-stranded nucleic acid–binding proteins in *D. melanogaster,* were further characterized to confirm their authenticity as hnRNP proteins. One of the most stringent criteria for identifying hnRNP and mRNP proteins is in vivo crosslinking to poly(A)–containing RNA (van Eekelen et al., 1981; Mayrand et al., 1981; Dreyfuss et al., 1984a). Living, intact S2 cells were irradiated with UV-light, and the poly(A)–containing RNA was isolated by oligo(dT) selection in the presence of SDS and β-mercaptoethanol to eliminate the association of proteins not covalently bound to the RNA. After RNase digestion to release the crosslinked proteins, the proteins were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and probed with the mAb 4C2 which reacts with the hrp40 proteins (Fig. 7). The slight decrease in the relative mobility of the hrp40 proteins (lane +UVXL) is characteristic of crosslinked proteins and is caused by residual nucleotides that remain covalently bound to the proteins after nuclease digestion (Dreyfuss et al., 1984a). The specificity of the crosslinking is demonstrated by the lack of detectable signal in the -UVXL lane. These results indicate that hrp40 is crosslinked to poly(A)-containing RNA in vivo
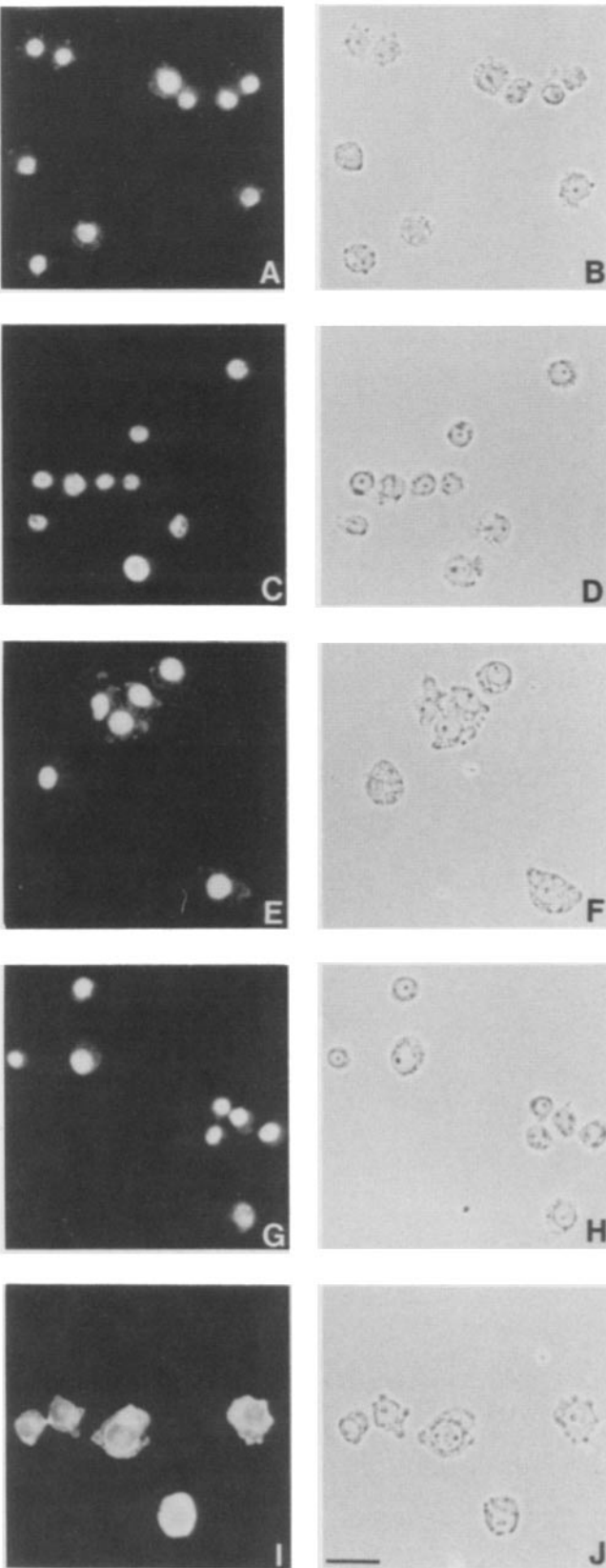
and, together with its nuclear localization, demonstrate that hrp40 is an authentic hnRNP protein. hrp36, hrp48, and hrp70 could not be shown to crosslink by similar analysis either because these proteins were not crosslinked efficiently by UV-light, or because the antibody recognition may have been abolished after crosslinking.

In addition, hrp40 was found to be immunologically related to several vertebrate hnRNP proteins. A two-dimensional immunoblot of HeLa cell hnRNP proteins, probed with mAb 4C2, demonstrates that hrp40 is related to the human A1, A2, B1, B2, and G proteins (Fig. 8, also see Piñol Roma et al., 1988). The sequence of the hrp40 proteins and their homology with the vertebrate A/B hnRNP proteins is discussed in the accompanying paper (Matunis et al., 1992).

Immunopurification of D. melanogaster hnRNP Complexes

In the presence of Empigen BB, an ionic detergent that disrupts protein-protein and protein-RNA interactions (Choi and Dreyfuss, 1984a), the mAb 8G6 specifically immunoprecipitates the hrp40 proteins (Fig. 9; *8G6*, lane *E*). In the absence of ionic detergents, however, 8G6 isolates a complex that contains hrp40 and many additional proteins (Fig. 9; *8G6*, lane *T*). The most prominent proteins detected by [³⁵S]methionine labeling are of 36, 38, 39, 40, 48, 62, and 100 kD. To determine whether the proteins that coimmunopurify with 8G6 are components of RNA-protein complexes, immunoprecipitations were also carried out after digestion with micrococcal nuclease (Fig. 9; *8G6*, lane *T/MN*). The sensitivity to RNase indicates that the complexes immunopurified with 8G6 contain RNA which is essential for the association of the protein components. No protein bands are detected when immunoprecipitations are carried out with SP2/0 ascites fluid (Fig. 9, lanes *SP2/0*), which were included as nonimmune controls.

The hnRNP complexes isolated with 8G6 were analyzed in detail by two-dimensional gel electrophoresis. Fig. 10 shows a comparison of 8G6 immunopurified hnRNP complexes and purified ssDNA-binding proteins from [³⁵S]methionine-labeled S2 cells. As is apparent by a comparison with Fig. 2, the major ssDNA-binding proteins detected by [³⁵S]methionine labeling are also the major proteins detected by Coomassie blue staining. In addition, most of the proteins that coimmunopurify with hrp40, including hrp36, hrp38, hrp39, hrp40, hrp44, hrp48, hrp54, hrp70, and hrp75 are among the major single-stranded nucleic acid-binding proteins in D. melanogaster. The nuclear localization of hrp36, hrp40, hrp44, and hrp70 (Fig. 5), and the staining of poly-

Figure 5. Immunofluorescence microscopy with antibodies 4C2, 5A5, 5C11, 3A, and 4A7. S2 cells were centrifuged onto glass slides, fixed, permeabilized, and stained with the following antibodies: *A* and *B*, 4C2 (anti-hrp40); *C* and *D*, 5A5 (anti-grp36); *E* and *F*, 5C11 (anti-hrp70); *G* and *H*, 3A (anti-hrp44); *I* and *J*, 4A7 (anti-mrp53). Immunofluorescence is shown on the left and the corresponding phase image on the right. Bar, 20 μm.

Figure 6. Immunofluorescent staining of polytene chromosomes with mAbs 8D2, 5A5, and 10D5. Salivary glands from third instar Oregon-R larvae were squashed in formaldehyde/acetic acid and probed with the following mAbs: *A* and *B*, 8D2 (anti-hrp40); *C* and *D*, 10D5 (hrp36); *E* and *F*, 5A5 (anti-hrp36). Fluorescence is shown on the left and the corresponding phase image on the right.
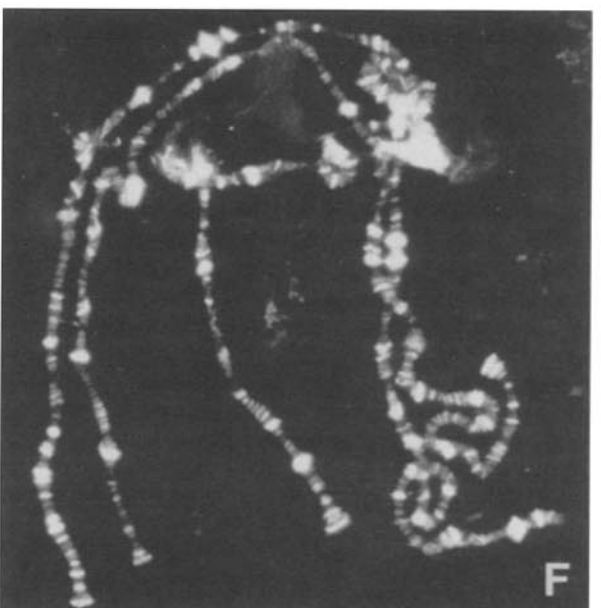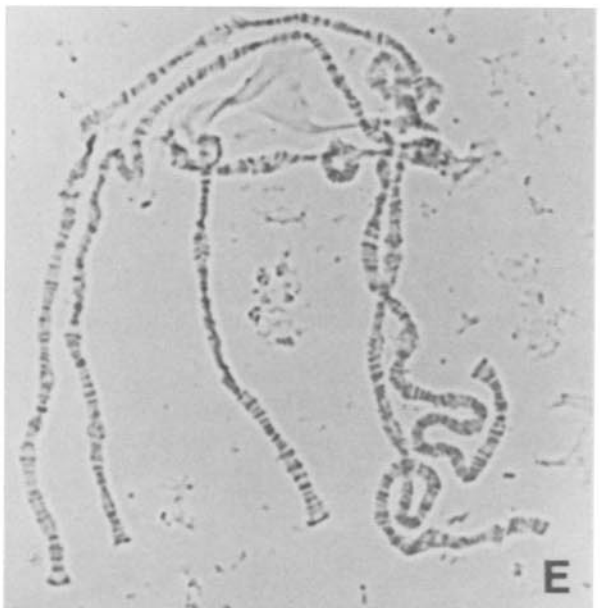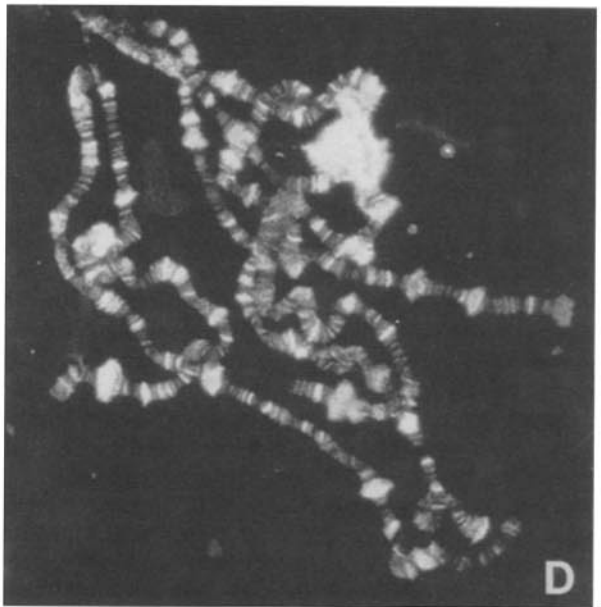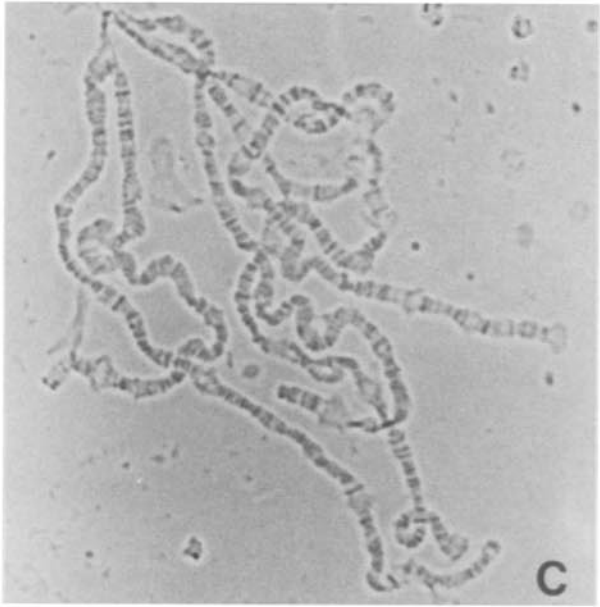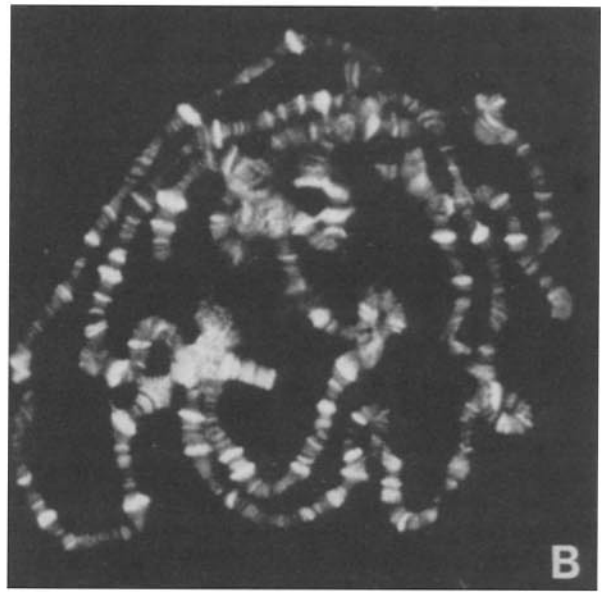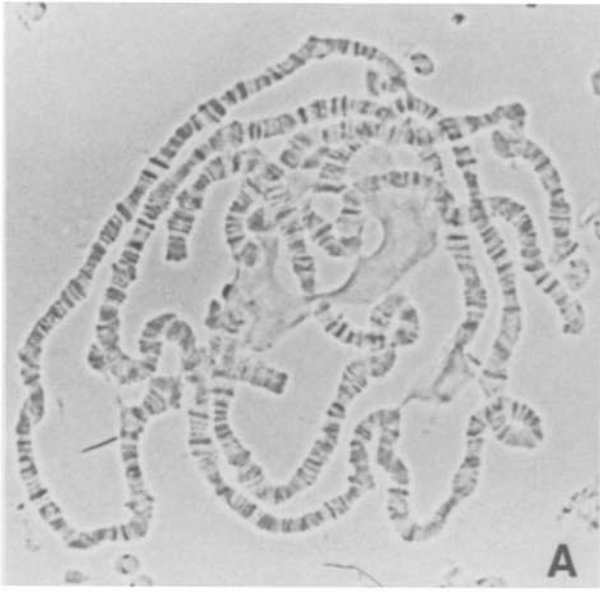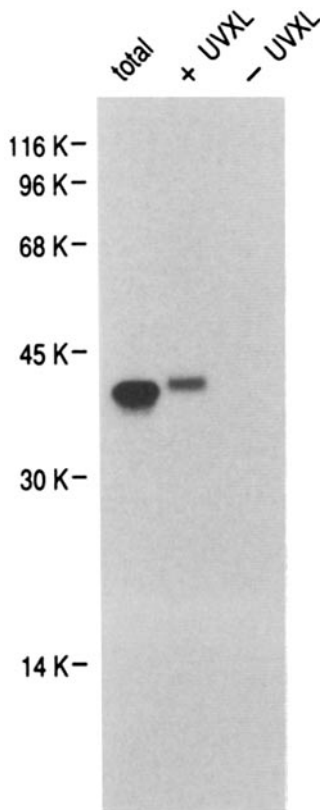
Figure 7. Immunoblot analysis of hrp40 crosslinked in vivo to poly(A)-containing RNA. S2 cells were irradiated with UV-light and poly(A)-containing RNA was isolated and digested with RNases. Released proteins were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and probed with the mAb 4C2. Lane *total*, total S2 cell proteins; lane *+UVXL*, proteins crosslinked to poly(A)-containing RNA; lane *−UVXL*, proteins from samples treated as in lane *+UVXL* except that cells were not irradiated with UV-light. Molecular weights of the protein standards are indicated.
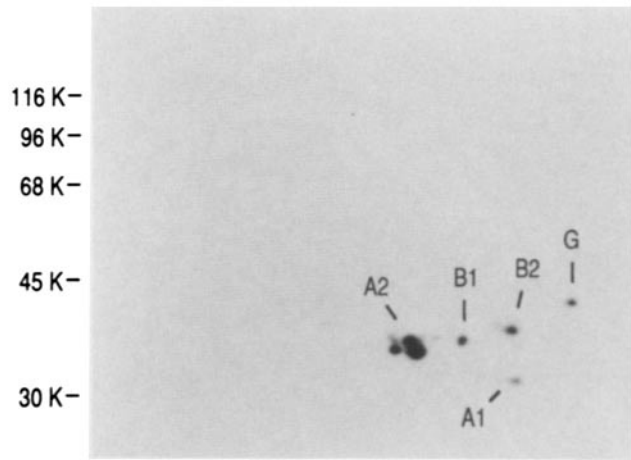

Figure 8. Two-dimensional immunoblot analysis of HeLa hnRNP proteins with the mAb 4C2. HeLa hnRNP proteins were purified by ssDNA chromatography and resolved by two-dimensional gel electrophoresis. Proteins were transferred to nitrocellulose membrane and probed with mAb 4C2. Proteins bound by 4C2 are indicated. Molecular weights of the protein standards are indicated.

tene chromosomes with antibodies to hrp36, hrp40, and hrp48 (Fig. 6), are further evidence that these proteins, which coimmunopurify with hrp40, are genuine hnRNP proteins. The specificity of the immunopurification procedure is also supported by the fact that cytoplasmic RNA-binding proteins, which are present in the cell lysate used, are not found in 8G6-immunopurified hnRNP complexes. For example, mrp53 is detected predominantly in the cytoplasm with mAb 4A7 (Fig. 5, *I* and *J*), and is absent from complexes immunopurified with 8G6. There are also several proteins, most notably hrp62, which do not bind ssDNA, but which are abundant components of immunopurified complexes. Similarly, several vertebrate hnRNP proteins do not bind ssDNA (e.g., Fig. 5, *H* and *F*), although they bind RNA (Piñol Roma et al., 1988; Swanson and Dreyfuss, 1988). Furthermore, a complex of similar composition is immunopurified with the mAb 5A5, although not as efficiently as with 8G6 (data not shown). Many of the mAbs obtained, however, including other antibodies to hrp36 and hrp40, do not immunopurify complexes possibly as a result of the inaccessibility of particular epitopes.

Discussion

In this study, we have isolated hnRNP complexes and characterized the major hnRNP proteins from *D. melanogaster*. Taking advantage of the fact that hnRNP proteins are the most abundant single-stranded, nucleic acid–binding proteins in vertebrates, candidate hnRNP proteins were purified from *D. melanogaster* embryos by ssDNA chromatography. mAbs were produced to many of these proteins and several

criteria were used to definitively identify genuine hnRNP proteins. The criteria we have used include: association with RNase-sensitive complexes, nucleoplasmic localization, association with transcriptionally active loci on polytene chromosomes, UV crosslinking to poly(A)-containing RNA in vivo, immunological relatedness to vertebrate hnRNP proteins, and predicted amino acid sequence (see Matunis et al., 1992).
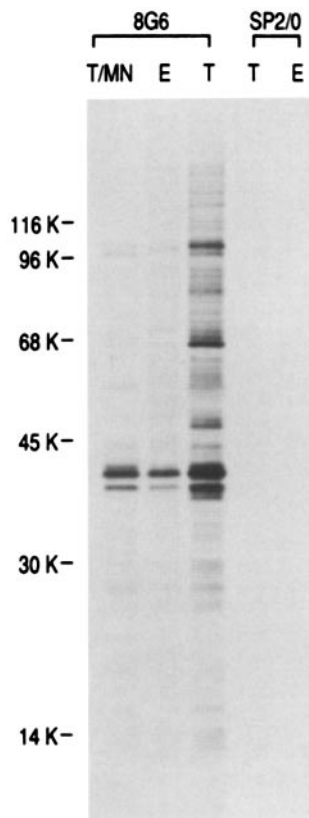

Figure 9. hnRNP complexes immunopurified with mAb 8G6. hnRNP complexes were immunopurified from [35S]-methionine-labeled S2 lysate in the presence of 0.5% Triton X-100 (lanes *T*) with the monoclonal antibody 8G6. To illustrate the RNase sensitivity of these complexes, samples were predigested with 100 U/ml micrococcal nuclease before immunoprecipitation in the presence of Triton X-100 (lane *T/MN*). Antibody specificity was demonstrated by immunopurification in the presence of 1% Empigen BB (lanes *E*). Control immunoprecipitations were with ascites fluid from a BALB/c mouse inoculated with the parent myeloma line SP2/0. Molecular weights of the protein standards are indicated.
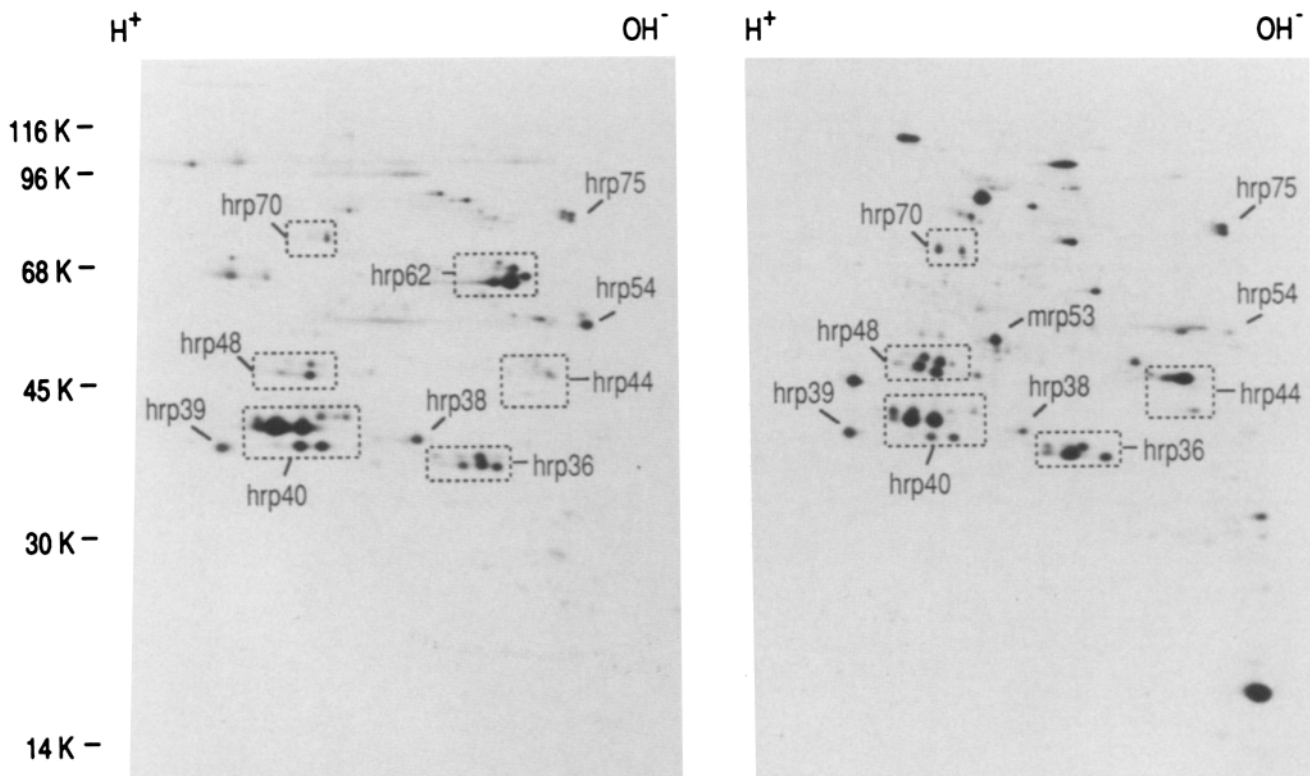
Figure 10. Comparison of 8G6 immunopurified hnRNP complexes and ssDNA-cellulose–purified proteins by two-dimensional gel electrophoresis. Proteins were purified from [^{35}S]methionine-labeled S2 lysate either by immunopurification with the monoclonal antibody 8G6 (*left panel*) or by ssDNA chromatography (*right panel*). ssDNA purified proteins were eluted from ssDNA-cellulose at 2 M NaCl after a 1-mg/ml heparin wash. The identity of the major proteins and the molecular weights of the protein standards are indicated.

Although many attempts have been made to analyze hnRNP complexes from *D. melanogaster* (Beyer et al., 1981; Christensen et al., 1981; Szabo et al., 1981; Wooley and Chung, 1981; Dangli et al., 1983; Kloetzel and Bautz, 1983; Mayrand and Pederson, 1983; Risau et al., 1983; Glatzer and Kloetzel, 1986; Kloetzel and Schuldt, 1986), these complexes have not previously been purified. Sucrose gradient sedimentation studies have suggested that the hnRNP complexes of *D. melanogaster* may be similar to those of vertebrates, but high nuclease activities present in *D. melanogaster* cell lines and embryos have made it difficult to isolate hnRNP complexes using this methodology (Matunis, M., and G. Dreyfuss, unpublished results; Szabo et al., 1981; Wooley and Chung, 1981; Risau et al., 1983). In addition, the interpretation of the cosedimentation of RNA and proteins across gradients is difficult, as is apparent from the conflicting reports on the effect of heat shock on *D. melanogaster* hnRNP complexes (Mayrand and Pederson, 1983; Kloetzel and Schuldt, 1986). The isolation of hnRNP complexes by immunopurification is a rapid procedure (thus minimizing exposure to RNases) and is highly specific by virtue of the monoclonal antibodies used. Antibodies to hrp40 have allowed us to immunopurify hnRNP complexes from *D. melanogaster* S2 cells. These hnRNP complexes, like those of vertebrates (Lothstein et al., 1985; Choi and Dreyfuss, 1984*a*), require RNA for their integrity as demonstrated by their sensitivity to digestion with RNase. Taking into account the many isoforms and numerous less abundant proteins, *D. melanogaster* hnRNP complexes have a diversity of

proteins similar to that of vertebrate hnRNP complexes. However, the identification of hnRNP homologues between *D. melanogaster* and vertebrates is not possible based on the two-dimensional mobilities of these proteins. A better picture of the relationship between individual vertebrate and *D. melanogaster* hnRNP proteins emerges from antibody cross-reactivities and from the sequences of the proteins which are discussed in the accompanying paper (Matunis et al., 1991). We also note that the major hnRNP proteins, including hrp40, are similar in molecular weight to the nuclear proteins crosslinked to poly(A)-containing RNA in living *D. melanogaster* cells (Kloetzel and Bautz, 1983).

Because invertebrate hnRNP complexes have not previously been purified, there has been speculation about their composition. For example, an hnRNP protein (HD40) has been purified from the brine shrimp, *Artemia salina*, and has been proposed to be the single major component of hnRNP complexes in that organism (Thomas et al., 1981, 1983). In light of our present findings, however, we anticipate that hnRNP complexes across eukaryotes are composed of many RNA-binding proteins. The mAbs 4C2 (this paper; Matunis et al., 1992) and 7A9 (Matunis, M., S. Piñol Roma, and G. Dreyfuss, manuscript submitted for publication), which recognize hnRNP proteins from yeast to man, should be useful for isolating hnRNP complexes from a wide range of organisms and provide a better understanding of the composition and evolution of these complexes.

As in vertebrates, the question remains as to why such a large number of hnRNP proteins exist in *D. melanogaster.*

Ribohomopolymer binding and Northwestern blotting experiments indicate that several of the *D. melanogaster* hrps have different RNA-binding activities, as found in vertebrates (Matunis, M., and G. Dreyfuss, data not shown; Swanson and Dreyfuss, 1988). This specialization could explain, in part, the need for such a large assortment of hnRNA-binding proteins. The ability to analyze protein function by genetic means offers the potential to test for functional redundancy among the many different hnRNP proteins or for subtle, essential differences in their binding activities. The large number of structurally similar hnRNP proteins in *D. melanogaster* and in vertebrates (e.g., A/B-like proteins, see accompanying paper) raises the possibility that several of them may have overlapping functions. On the other hand, the binding of different hnRNP protein isoforms to specific RNAs could affect pre-mRNA processing in essential ways.

hnRNP complexes isolated by immunopurification with the mAb 8G6 represent the totality of hnRNA-protein complexes containing hrp40 and do not address the existence of complexes not containing hrp40 (if such complexes exist) or of transcript-specific protein complexes. Hrp40 and the other major hrps localize to most, if not all, transcriptionally active loci and we thus assume that they are common to the majority of the transcripts. The possibility that some of the minor proteins observed in 8G6-immunopurified hnRNP complexes are transcript-specific proteins is important in light of the many examples of alternative pre-mRNA processing (for review see Latchman, 1990). Many of the minor proteins which are consistently present in the immunopurified complexes isolated with 8G6 could be proteins which are present in a lower ratio per complex, or associated with only a subset of hnRNAs. Several sex-specific and tissue-specific *D. melanogaster* proteins that appear to function as transcript-specific regulators of alternative splicing have, in fact, been identified. Three of these proteins, the gene products of *sxl*, *tra2*, and *elav*, have been shown to contain RNP-consensus sequence RNA-binding domains, indicative of RNA-binding activity (Amrein et al., 1988; Bell et al., 1988; Dreyfuss et al., 1988; Robinow et al., 1988; Bandziulis et al., 1989; Goralski et al., 1989; Mattaj, 1989; Query et al., 1989). Several other transcript-specific factors may include the *suppressor-of-white-apricot* gene product and several proteins that likely regulate tissue-specific P element processing (Zachar et al., 1987; Chou et al., 1987; Siebel and Rio, 1990). At least some of these factors fulfill the criteria of hnRNP proteins in that they interact with hnRNA directly and they do not appear to be stable components of snRNPs (Inoue et al., 1990; Hedley and Maniatis, 1991; Hoshijima et al., 1991). Their possible association with hnRNP complexes is therefore of interest, and it should now be possible to address this issue with the immunopurification of these complexes. The study of transcript-specific protein complexes will also be facilitated by the ability to directly visualize the association of hnRNP proteins on the nascent transcripts of polytene chromosomes. Similar analysis on the amphibian oocyte lampbrush chromosomes illustrated the ability of certain hnRNP proteins to discriminate among nascent transcripts. For example, the vertebrate hnRNP L protein binds to most nascent transcripts but, in particularly large amounts to the transcripts of the giant loops locus to which other known hnRNP proteins do not bind (Piñol Roma et al., 1989). The more defined transcription units of *D. me-lanogaster* polytene chromosomes, in addition to the availability of the antibodies described here, should enable a detailed characterization of the transcript-binding properties of individual hnRNP proteins.

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