Association of Individual hnRNP Proteins and snRNPs with Nascent Transcripts

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Abstract. As they are transcribed, RNA polymerase II transcripts (hnRNAs or pre-mRNAs) associate with hnRNP proteins and snRNP particles, and the processing of pre-mRNA occurs within these ribonucleoprotein complexes. To better understand the relationship between hnRNP proteins and snRNP particles and their roles in mRNA formation, we have visualized them as they associate with nascent transcripts on the polytene chromosomes of *Drosophila melanogaster* salivary glands. Simultaneous pairwise detection of the abundant hnRNP proteins hrp36, hrp40, and hrp48 by direct double-label immunofluorescence microscopy reveals all of these proteins are bound to most transcripts, but their relative amounts on different transcripts are not fixed. Numerous differences in the relative amounts of snRNP particles and hnRNP proteins on nascent transcripts are also observed. These observations directly demonstrate that individual hnRNP proteins and snRNP particles are differentially associated with nascent transcripts and suggest that different pre-mRNAs bind different combinations of these factors to form transcript-specific, rather than a single type of, hnRNA-hnRNP-snRNP complexes. The distinct and specific constellation of hnRNP proteins and snRNP particles that assembles on different pre-mRNAs is likely to affect the fate and pathway of processing of these transcripts.

N ASCENT RNA polymerase II transcripts (hnRNAs or pre-mRNAs) associate with both hnRNP proteins and snRNP particles, and it is within these hnRNA-hnRNP-snRNP assemblies that the processing of pre-mRNA occurs. Although hnRNP proteins and snRNPs interact with pre-mRNAs and are involved in their processing (for review see Dreyfuss, 1986; Maniatis and Reed, 1987; Dreyfuss et al., 1988; Lührmann et al., 1990; Guthrie, 1991; Green, 1991), the interactions between them are not well defined. Much of the current knowledge about hnRNP complexes comes from studies of vertebrate cells, particularly HeLa cells, which contain over 20 abundant nuclear hnRNA-binding proteins (Piñol-Roma et al., 1988). As these proteins are likely to influence the structure of pre-mRNAs and participate in a variety of RNA processing events (for review see Bandziulis et al., 1989), the study of their specific RNA binding properties and functions is of great interest. Toward this goal, we have recently isolated hnRNP complexes from *Drosophila melanogaster*, and found they are composed of at least 10 abundant proteins associated with hnRNAs (hrp's; Matunis et al., 1992a). We have also generated mAbs to several of these proteins (Matunis et al., 1992a) and have characterized them at the molecular level (Matunis et al.,

1992b). All of the abundant *D. melanogaster* hnRNP proteins characterized thus far are similar in primary structure to the vertebrate A/B hnRNP proteins, with two amino-terminal RNA-binding domains and a glycine-rich carboxyl-terminal domain, or 2×RBD-Gly (Matunis et al., 1992b; Raychaudhuri et al., 1992).

The polytene chromosomes of *D. melanogaster* provide an excellent system in which to observe the proteins associated with specific transcriptional loci in situ. DNA-binding proteins such as histones, as well as nonhistone chromosomal proteins (Desai et al., 1972; Silver and Elgin, 1976; Alfageme et al., 1976; Saumweber et al., 1980), and RNA-binding proteins have been visualized in this manner (Kabisch and Bautz, 1983; Risau et al., 1983; Matunis et al., 1992a). On spread polytene chromosomes, the associations of specific proteins with RNA can be observed during transcription and processing, and this can provide important information about hnRNA-hnRNP-snRNP interactions at the earliest stages of mRNA formation. Complexes at these stages are likely to be distinct from soluble nucleoplasmic post-chromatin protein-RNA complexes obtained by methods such as sucrose gradient centrifugation and immunopurification (e.g., Beyer et al., 1977; Wilk et al., 1985; Piñol-Roma et al., 1988), and they may be more relevant to pre-mRNA processing which begins on the nascent transcripts (Beyer and Osheim, 1988).

Here, we have used mAbs to several of the major *D. me-*

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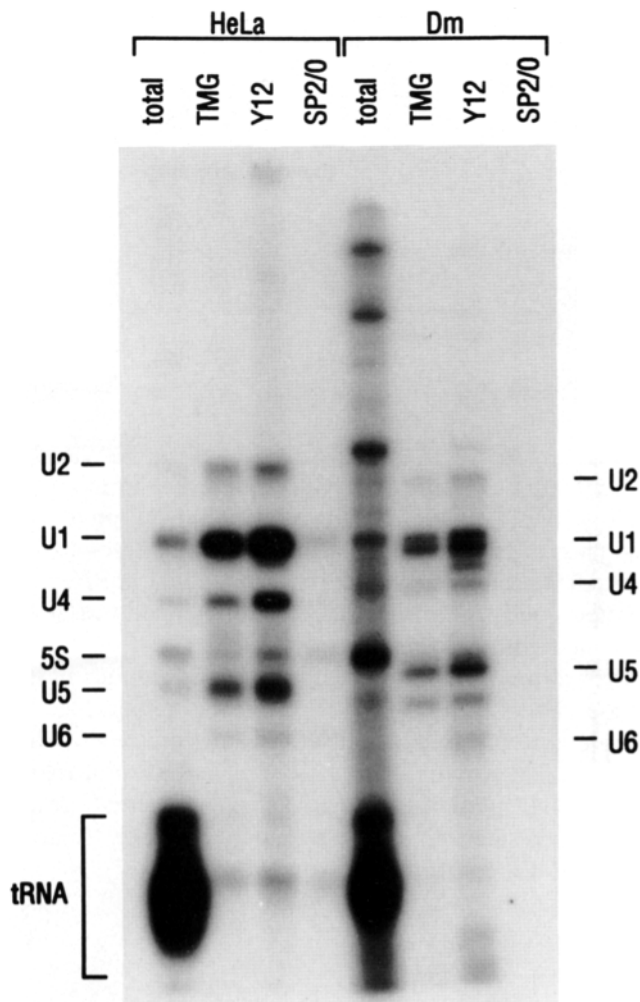
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Figure 1. Immunopurification of HeLa and *D. melanogaster* snRNAs. snRNAs were immunoprecipitated from nuclear extract with an anti-trimethylguanosine cap mAb (lane *TMG*) or with the anti-Sm mAb Y12 (lane *Y12*). RNAs were 3'-end labeled in vitro using T4 RNA ligase and separated by electrophoresis on a 7-M urea-10% polyacrylamide gel. As controls, total RNA from nuclear extract and RNA precipitated with ascites fluid from a mouse inoculated with the parental myeloma cell line, SP2/0, was labeled (lanes *total* and *SP2/0*). The probable positions of the major snRNAs are indicated. Positions of 5S and tRNAs are also indicated.

Figure 2. Immunological comparison of *D. melanogaster* and HeLa snRNP proteins with the anti-Sm mAb Y12. Total HeLa and *D. melanogaster* cell lysates were separated by SDS-PAGE, transferred to nitrocellulose, and probed with the mAb Y12. snRNP B, B', and D proteins, and the molecular masses of protein standards are indicated.

Materials and Methods

Gel Electrophoresis and Immunoblotting

Electrophoresis of proteins in SDS-containing discontinuous polyacrylamide gels (SDS-PAGE) was performed as described previously (Dreyfuss et al., 1984) using 15% polyacrylamide in the separating gel. Immunoblotting was done as described previously (Choi and Dreyfuss, 1984), using Y12 ascites fluid at a 1:1,000 dilution.

Cell Culture and Preparation of Nuclear Extracts

Schneider's line 2 *D. melanogaster* cells (S2) were cultured at 25°C in modified Schneider's *D. melanogaster* medium (Gibco BRL, Gaithersburg, MD) supplemented with 10% FCS, penicillin, and streptomycin. Human HeLa cells were cultured at 37°C in DME supplemented with 10% calf serum, penicillin, and streptomycin. HeLa nuclear extracts were prepared as described by Dignam et al. (1983). Schneider's nuclei were isolated by lysing the cells in Dignam buffer A containing 0.5% NP-40. Cells were allowed to swell for 15 min on ice, vortexed, and nuclei were pelleted by centrifugation for 5 min at 5,000 g. Nuclear extract was then prepared according to Dignam et al. (1983).

Immunopurification and Labeling of snRNAs

For immunopurification of snRNAs, mAbs were bound to protein A-Sepharose (Pharmacia LKB Biotechnology, Piscataway, NJ) for 1 h in PBS, and then washed two times with buffer A (RSB-100 containing 0.5% Triton X-100, 1% aprotinin, 2 μg/ml leupeptin, and 2 μg/ml pepstatin A). The antibody-protein-A beads were resuspended in 0.5 ml of buffer A, and 50 μl of nuclear extract was added and incubated for 30 min at 4°C with mixing. Antibody complexes were washed five times with buffer A, resuspended in 75 μl of TE containing 0.1% SDS and boiled for 3 min. After boiling, 225 μl of TE was added to each tube, the protein A-Sepharose beads were pelleted, and the supernatant was removed and extracted two times with PCA (phenol, chloroform, isoamylalcohol, 25:24:1). RNAs were precipitated with ethanol and 3' end labeled with T4 RNA ligase as described by England et al. (1980). RNAs were analyzed by electrophoresis through 10% polyacrylamide-7 M urea gels. Immunopurifications were also carried out using ascites fluid from a mouse inoculated with the SP2/0 myeloma cell line as a control.

lanogaster hnRNP proteins (Matunis et al., 1992a) to simultaneously visualize their distribution on nascent transcripts by direct immunofluorescence and laser scanning confocal microscopy. We find that although a large number of nascent transcripts contain all of the different hnRNP proteins analyzed here, the relative amounts of these proteins vary on different transcripts. Furthermore, simultaneous detection of hnRNP and snRNP proteins reveals that these two types of RNA-binding components are also differentially associated with pre-mRNAs. These findings demonstrate that the individual hnRNP proteins and snRNP particles are not present on nascent transcripts in a fixed ratio. Rather, hnRNP proteins and snRNP particles can interact with RNA independently and differentially, and they appear to associate with individual pre-mRNAs in a transcript-specific manner.
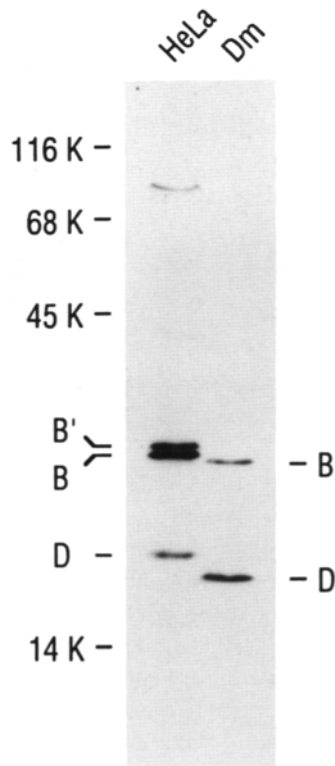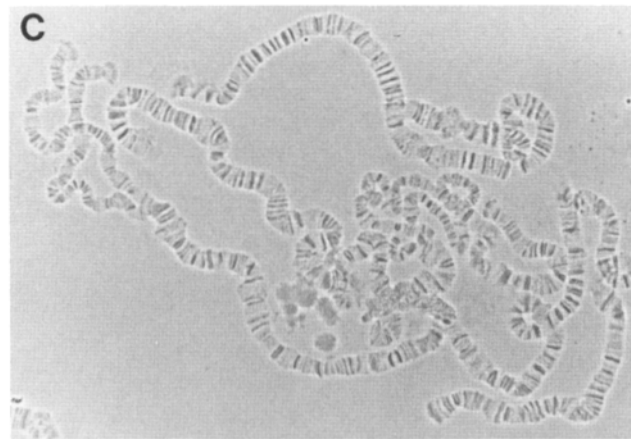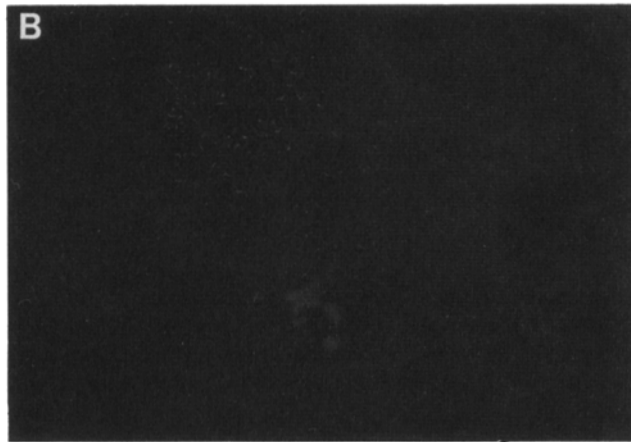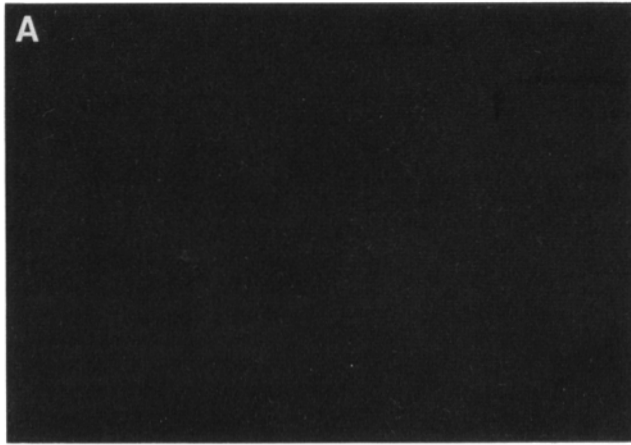
Figure 3. RNAase sensitivity of the association of hnRNP proteins and snRNPs with nascent transcripts. Salivary gland polytene chromosomes from third instar larvae were pretreated with RNAase A and stained with a monoclonal anti-hrp36 antibody directly labeled with Texas red (*A*), and the monoclonal anti-snRNP antibody Y12, which was directly labeled with FITC (*B*). The corresponding phase contrast image is in *C*.
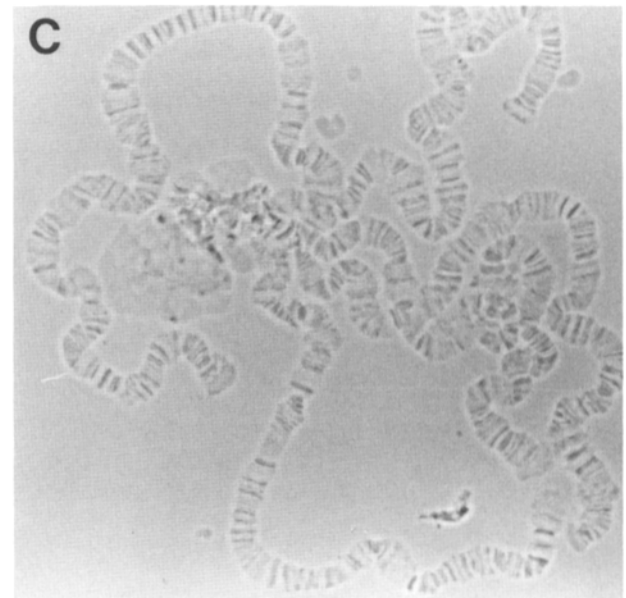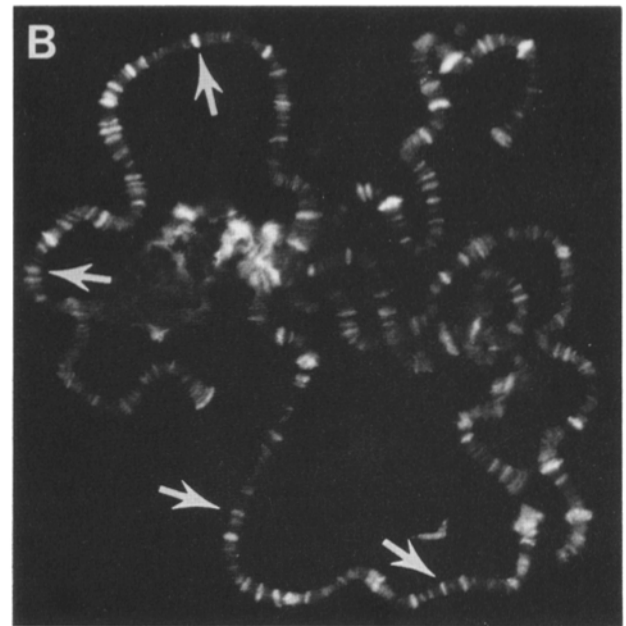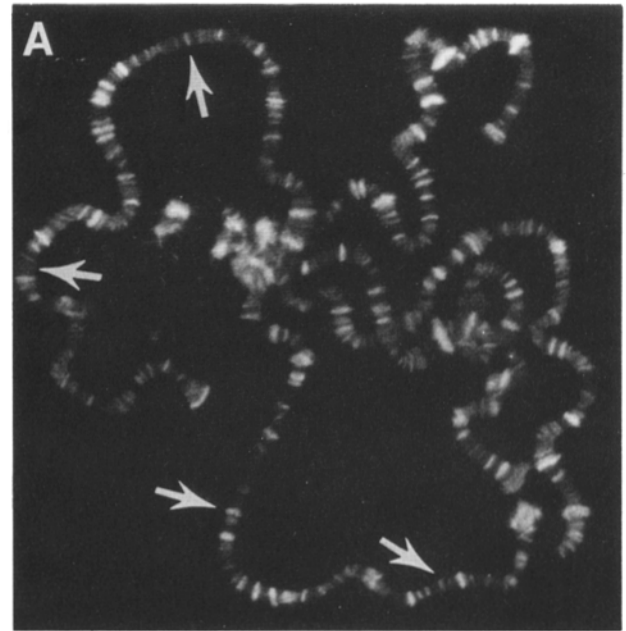
Figure 4. Simultaneous localization of the *D. melanogaster* hnRNP proteins hrp36 and hrp48 on nascent transcripts. Salivary gland polytene chromosomes from third instar larvae were stained with anti-hrp36 which was directly labeled with Texas red (*A*), and anti-hrp48 which was directly labeled with FITC (*B*). Loci displaying relative differences in the intensity of staining with these two antibodies are denoted with arrowheads. The corresponding phase contrast image for this field is in *C*.

hrp36   **hrp48**   **combined**

Figure 5. Simultaneous visualization of hrp36 and hrp48 on nascent transcripts. Double-label immunofluorescence using anti-hrp antibodies directly labeled with Texas red (*hrp36, A*) and FITC (*hrp48, B*) on salivary gland polytene chromosomes from third instar larvae. The confocal micrographs in *A* and *B* are depicted in a color scheme which represents a pseudo-color intensity map of the signals, ranging from blue (*low intensity*) to turquoise, green, yellow, red, and white (*high intensity*). Loci displaying relative differences in the intensity of staining with these two antisera are denoted with arrowheads. In *C*, the signals from anti-hrp36 (*green*) and anti-hrp48 (*red*) have been merged. Loci which are bound by relatively more hrp36 than hrp48 appear green or greenish yellow, while loci at which there is more hrp48 than hrp36 appear red or reddish yellow. Areas in which the relative intensity of these two signals is similar are yellow, which is the case for one site in this field.

Preparation of Directly Labeled mAbs

mAbs 5A5, 10D5, and 4C2 have been described in detail (Matunis et al., 1992a). Anti-2,2,7-trimethylguanosine (TMG)[1] was obtained from Oncogene Science Inc. (Manhasset, NY). The anti-Sm mAb Y12 (Lerner et al., 1981; Pettersson et al., 1984) was a kind gift of Dr. Joan Steitz. Antibodies were purified from mouse ascites fluid by HPLC on a polyethylene-imine column (Rainin Instrument Co. Inc., Woburn, MA) as suggested by the manufacturer. Antibodies were conjugated to fluorescein isothiocyanate as described (Harlow and Lane, 1988) or to Texas red as described (Titus et al., 1982).

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). RNAase A-treated chromosomes were prepared in the same manner, except RNAase A (100 μg/ml final concentration) was added to the medium G. For heat shock experiments, glands were dissected from larvae incubated 15 min at 37°C. Before freezing, the slides were sealed with rubber cement and photographed under phase contrast. For immunostaining, directly labeled mAbs were diluted in PBS(−) containing 3% BSA and 0.02% sodium azide, and 20 μl of each diluted antibody solution was placed on a coverslip. The pairs of antibodies were mixed by pipetting, and the coverslip was 'picked up' with a slide containing squashed chromosomes. After incubation for 1 h at room temperature in a humid chamber, the slides were rinsed three times in PBS(−), mounted in 100% glycerol containing 5% n-propyl gallate, 0.25% 1.4-diazabicyclo-[2.2.2]octane (DABCO; triethylenediamine) and 0.0025% p-phenylenediamine, and photographed on Kodak T-max 400 film using a Zeiss Axiophot, or on Kodak Ektachrome 100HC film using a laser scanning confocal microscope (model MRC600; BioRad Labs., Hercules, CA). Efficient separation of the fluorochromes between the two channels was achieved using the 476- and 568-nm lines from a Krypton laser and a set of filters designed for this purpose (Omega Optical Inc., Brattleboro, VT).

Results

Specificity of the Antibodies to D. melanogaster hnRNP Proteins and snRNP Particles

The specificities of the mAbs to *D. melanogaster* hnRNP

1. *Abbreviation used in this paper:* TMG, anti-2,2,7-trimethylguanosine.

proteins used in this study have been described in detail recently (Matunis et al., 1992a). To visualize snRNPs we have used the mAb Y12 which is specific for the Sm epitope common to the five major splicing snRNPs (Lerner et al., 1981). Although the specificity of this antibody for the mammalian snRNP particles (by recognition of several polypeptides common to the U1, U2, U4/U6, and U5 snRNPs) has been documented (Lerner et al., 1981; Pettersson et al., 1984), the reactivity of this antibody towards *D. melanogaster* snRNP proteins has not been analyzed in detail. Therefore, we compared ribonucleoprotein complexes immunopurified from *D. melanogaster* Schneider cell nuclear extracts with Y12 and anti-TMG, (a mAb specific for the TMG cap [Krainer, 1988], a unique feature of the U1, U2, U4, and U5 snRNAs). The Y12 and the anti-TMG mAbs immunopurified nearly identical RNAs from *D. melanogaster* nuclear extracts (Fig. 1; Dm, lanes *Y12* and *TMG*). These RNAs are very similar in size to previously reported *D. melanogaster* snRNAs (Mount and Steitz, 1981), and are also similar in size to the snRNAs immunopurified in parallel from human HeLa nuclear extracts (Fig. 1; HeLa, lanes *Y12* and *TMG*) (Lerner et al., 1981; Pettersson et al., 1984).

The antigenic determinants recognized by Y12 in humans reside on several polypeptides that are common to the U1, U2, U4/U6, and U5 snRNPs, specifically, the B, B′, and D proteins (Pettersson et al., 1984). To further verify the specificity of Y12 in *D. melanogaster*, an immunoblot of total HeLa cell and *D. melanogaster* cell lysates was probed (Fig. 2). Two *D. melanogaster* polypeptides are recognized by Y12, one slightly smaller than the human D protein (16 kD), and one approximately the same size as the human B protein (28 kD). Based on their size and antigenicity, these *D. melanogaster* proteins are likely to be the counterparts of the human B and D proteins (Paterson et al., 1991). Importantly, the Y12 mAb does not react with other *D. melanogaster* proteins and it thus appears to be specific for snRNP proteins.

In the experiments described here, the proteins detected

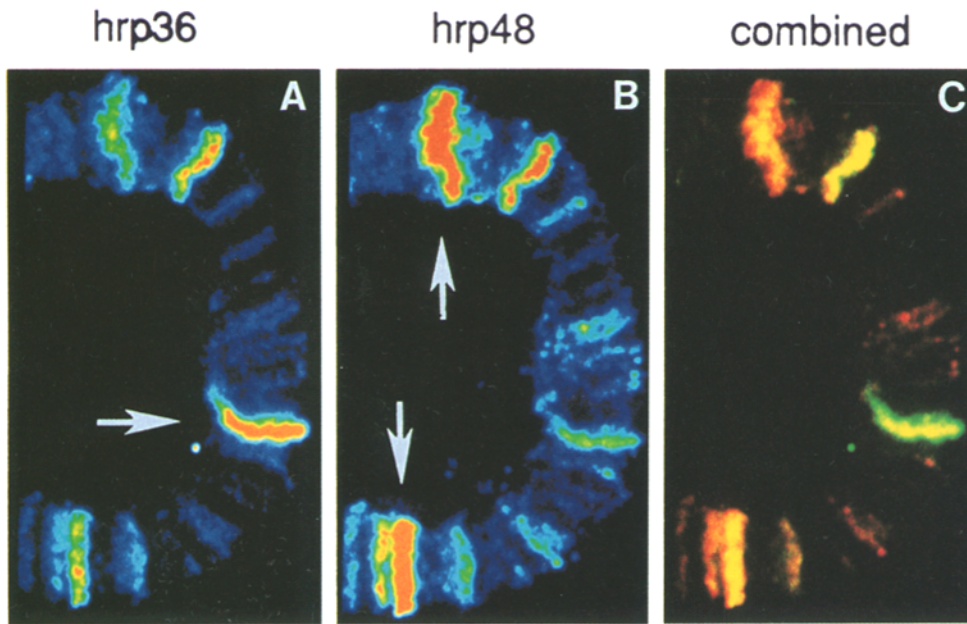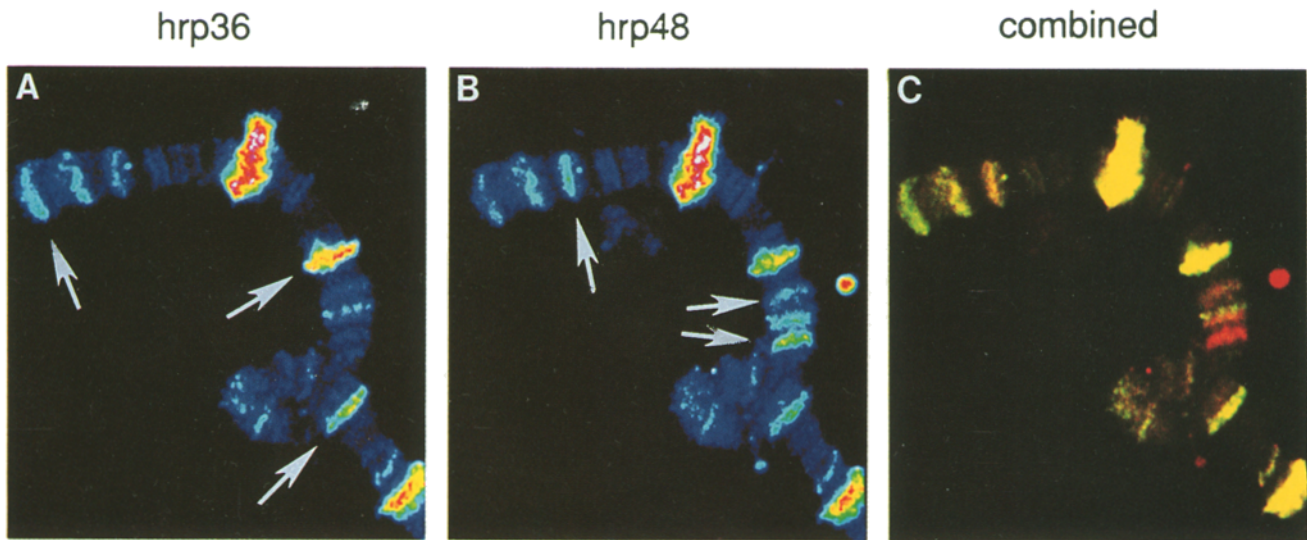hrp36   hrp48   combined

Figure 6. Simultaneous localization of hrp36 and hrp48 on nascent transcripts. Double-label immunofluorescence on salivary gland poly-
tene chromosomes using monoclonal anti-hrp antibodies directly labeled with Texas red (*hrp36, A*) and FITC (*hrp48, B*). The confocal
micrographs in *A* and *B* are depicted in the color scheme described in the legend for Fig. 5. In *C*, the signals from anti-hrp36 (*green*)
and anti-hrp48 (*red*) in this field have been merged.

immunologically on polytene chromosomes are associated
with RNA, rather than with chromatin, because no hrp36 or
snRNP signal is detectable in salivary gland squashes that
were treated with RNAase A before fixation and preparation
(Fig. 3; *A* and *B*). The same result was obtained using anti-
hrp40 and anti-hrp48 antibodies (data not shown). Hence,
the immunofluorescence signals seen in Figs. 4 through 11
result from the detection of proteins that are bound to
chromatin-associated RNAs. A very low level of residual
staining from the anti-hnRNP or anti-snRNP antibodies is
sometimes observed near the chromocenter (e.g., Fig. 3 *B*),
which may be less susceptible to RNAase A digestion. The
integrity of the chromatin is not affected by the RNAase
treatment, as shown in the phase contrast image (Fig. 3 *C*).

Simultaneous Visualization of
the Major hnRNP Proteins hrp36, hrp40, and hrp48
on Nascent Transcripts

To study the distribution of individual hnRNP proteins on
different nascent transcripts of *D. melanogaster* polytene
chromosomes, we carried out simultaneous immunolocali-
zation of hnRNP proteins using monoclonal antibodies
specific for hrp36 (5A5) and hrp48 (10D5) which were
directly labeled with the nonoverlapping fluorophores Texas
red or fluorescein isothiocyanate, respectively (Fig. 4).
Comparison of Fig. 4, *A* and *B* shows that the overall local-
ization patterns for hrp36 and hrp48 are very similar. Since
hrp36 and hrp48 are major components of *D. melanogaster*
hnRNP complexes, it is not surprising that these proteins are
bound to a large number of puffs and interbands (which con-
tain the units of transcriptional activity; for review see Dane-
holt, 1975). We note that some telomeric regions also appear
to be stained with the anti-hrp36 and anti-hrp48 antibodies
(Fig. 4). This staining is sensitive to RNAase A (Fig. 3), sug-
gesting that it is due to the association of hrp proteins with
RNA transcribed from these regions. The localization pat-

tern of the hrp36 and hrp48 proteins is similar to that seen
for RNA polymerase II (Plagens et al., 1976; Jamrich et al.,
1977; Kramer et al., 1980; Sass, 1982). However, even at
this level of resolution the distribution of these proteins is not
identical, as loci at which the relative amounts of these pro-
teins differ markedly can be detected (Fig. 4). Similar differ-
ences were consistently observed in multiple experiments
and on different preparations. The differential staining is not
due to differences in the affinities of the antibodies or the in-
tensities of the fluorophores since the relative signals from
the anti-hrp36 antibody are both stronger and weaker than
those from the anti-hrp48 antibody, and vice-versa. Further-
more, we have exchanged the fluorophores between pairs of
antibodies and observed similar results. The differences in
relative intensity of the two signals thus reflect the real differ-
ences in the relative amounts of the proteins bound to nascent
transcripts. In addition, when Texas red–conjugated anti-
hrp36 and FITC-conjugated anti-hrp36 were mixed and used
to visualize hrp36 on nascent transcripts, no differences in
the relative intensity of the signals were observed (not
shown). We cannot exclude the possibility that the differen-
tial staining may, in some cases, be the result of differential
accessibility of the antigens. Even if this is the case, it still
reflects a nonuniform arrangement of the individual proteins
on different transcripts. The formation of transcript-specific
hnRNP complexes has recently been observed in vitro (Ben-
net et al., 1992), and our data are consistent with this and
other previous in vitro studies of the association of hnRNP
proteins with pre-mRNA (see Discussion).

More detailed comparisons of the relative localization of
hrp36 and hrp48 on nascent transcripts were carried out by
use of laser scanning confocal microscopy which afford
higher resolution imaging and permits both a more quantita-
tive comparison of the signals and a more precise super-
imposition of the images. The simultaneous localization of
hrp36 and hrp48 is shown in Fig. 5, *A* and *B*, respectively,
and the superimposed signals are shown in Fig. 5 *C*. Specific
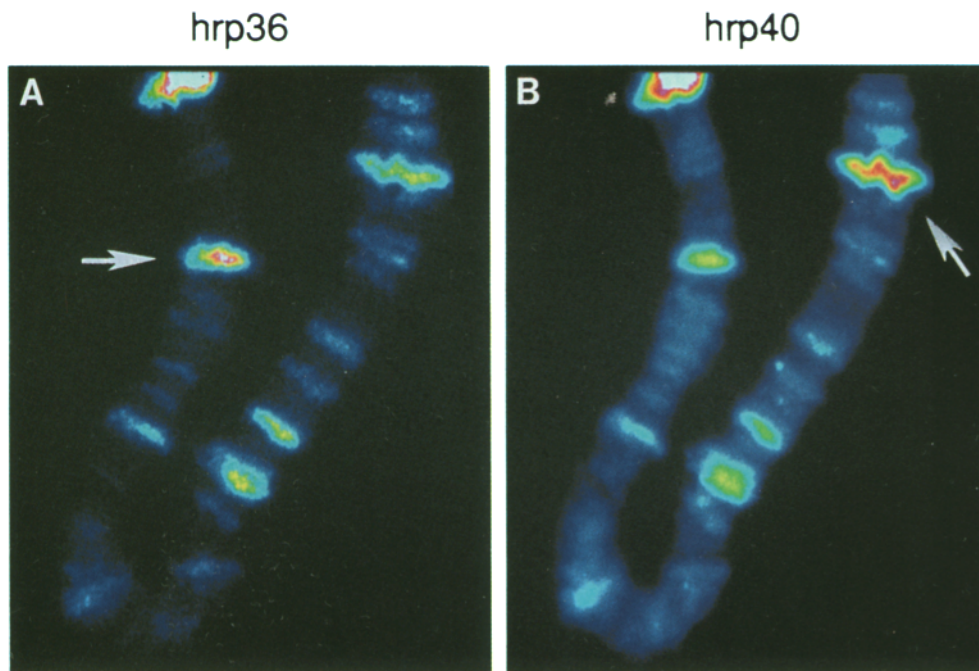loci are resolved where the relative amounts of these two pro-

hrp36   hrp40

Figure 7. Simultaneous visualization of hrp36 and hrp40 on nascent transcripts. Direct, double-label immunofluorescence using anti-hrp antibodies labeled with Texas red (*hrp36, A*) and FITC (*hrp40, B*) on salivary gland polytene chromosomes. The confocal micrographs are depicted in the color scheme described in the legend for Fig. 5. Loci displaying differences in the relative intensity of staining with these two antisera are denoted with arrowheads.

teins vary markedly, and examples of these are indicated with arrowheads. We estimate that at least ten percent of the loci contain markedly different relative amounts of these two proteins. The monoclonal anti-hrp antibodies each recognize several isoforms of the respective hrp protein. Therefore, differences in the binding of specific transcripts by proteins within a given hrp protein group cannot be detected using these antibodies. An additional field depicting the simultaneous visualization of hrp36 and hrp48 is shown (Fig. 6). This again demonstrates that different relative amounts of hrp36 and hrp48 are present on nascent transcripts. Comparison of the distribution of hrp36 and hrp40 also showed differences in the binding of these proteins to specific transcripts (Fig. 7, *A* and *B*, respectively).

To localize hnRNP proteins on readily identifiable well-characterized nascent transcripts, we took advantage of the heat shock response, which has been used as a model system for the study of inducible gene expression. Upon heat shock, nine loci encoding various heat shock proteins puff prominently. Two such heat-shock puffs are at the 87A and 87C loci, which encode the hsp70 protein (for review see Lindquist, 1986; Yost et al., 1990). The immunolocalization of hrp36 (Fig. 8 *A*) and hrp48 (Fig. 8 *B*) on the 87A and 87C loci (indicated with *arrowheads*) after heat shock is shown. It is apparent that the hsp70 pre-mRNAs bind hnRNP proteins. However, while hrp48 is readily detectable on these intronless transcripts after heat shock, there is relatively little hrp36 on these pre-mRNAs (Fig. 8). This experiment and those presented above demonstrates that although the abundant and highly related hnRNP proteins hrp36, hrp48, and hrp40 bind to most nascent transcripts, the relative amounts of these proteins vary on different transcripts.

Differential Association of D. melanogaster hnRNPs and snRNPs with Nascent Transcripts

Direct immunofluorescence with antibodies to hrp36 (Fig. 9 *A*) and snRNPs (Fig. 9 *B*) reveals that they colocalize on

most transcripts but shows that there are many loci at which the relative amounts of hnRNP proteins and snRNPs differ. Examples of some of these are indicated by arrowheads (Fig. 9). A similar number of differences is also observed when
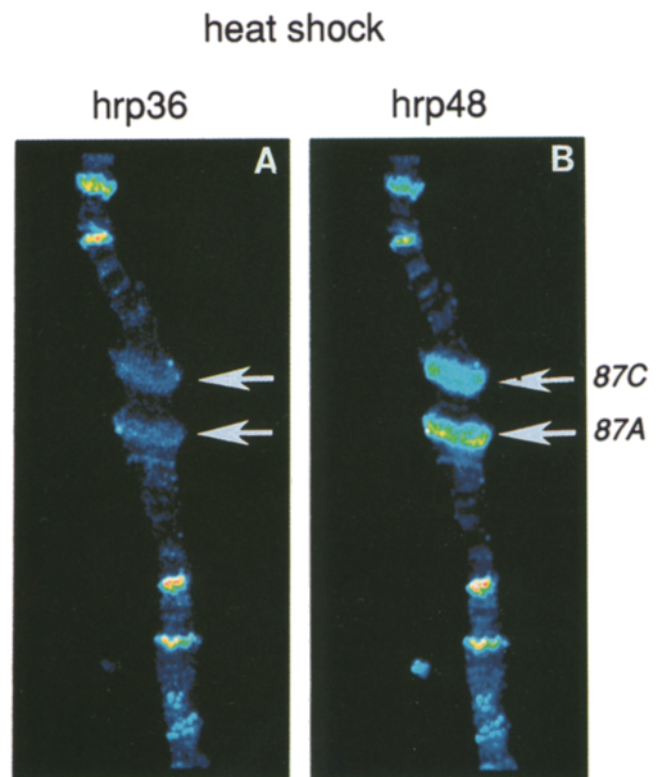
heat shock

hrp36   hrp48

87C
87A

Figure 8. Differential association of hrp36 and hrp48 at the 87A and 87C loci after heat shock. Double-label immunofluorescence on salivary gland polytene chromosomes using monoclonal anti-hrp antibodies directly labeled with Texas red (*hrp36, A*) and FITC (*hrp48, B*). The confocal images are depicted in the color scheme described in the legend for Fig. 5.
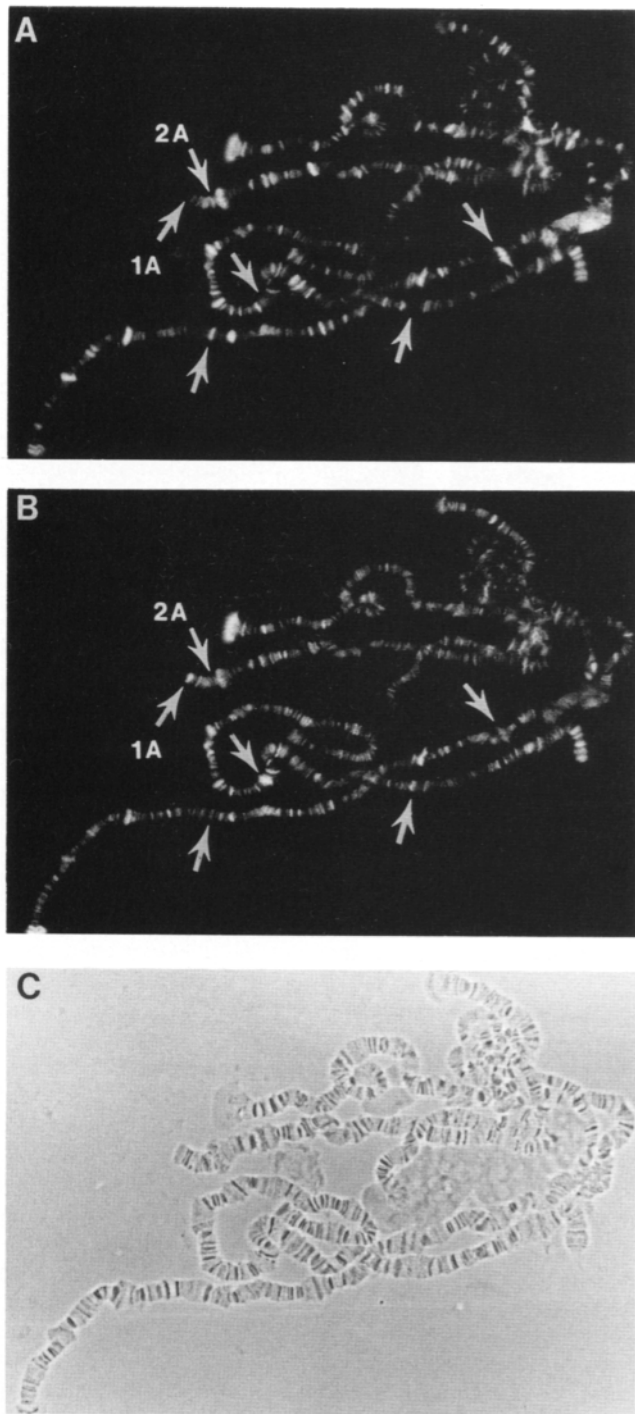
Figure 9. Simultaneous localization of hrp36 and snRNPs on nascent transcripts. Double-label immunofluorescence on salivary gland polytene chromosomes using a monoclonal anti-hrp36 antibody directly labeled with Texas red (*A*) and the monoclonal anti-snRNP antibody Y12, which was directly labeled with FITC (*B*). Some of the loci displaying differences in the relative intensity of staining with these two antisera are denoted with arrowheads (e.g., the 1A and 2A loci, which are indicated). The corresponding phase contrast image is in *C*.

antibodies to hrp40 or hrp48 are used simultaneously with the anti-snRNP antibody Y12 (not shown). The signal resulting from Y12 most likely represents snRNP–snRNA complexes, rather than free snRNP proteins, as snRNP proteins

must bind snRNA before their import into the nucleus (Mattaj and DeRobertis, 1985). Consistent with this, the distribution of snRNAs on polytene chromosomes visualized using an anti-TMG mAb is similar to that seen with Y12 (not shown). To further analyze the differences in the distribution of hnRNP and snRNP proteins, confocal micrographs of anti-hrp36 (Fig. 10 *A*) and anti-snRNP (Fig. 10 *B*) immunofluorescence were merged (Fig. 10 *C*). At this level of resolution, it is readily apparent that there are numerous significant differences in the relative amounts of these factors on many different nascent transcripts. The same ratios of hnRNP proteins and snRNPs on specific loci are consistently observed in numerous different preparations by both standard and confocal microscopy. For example, the same results are obtained when the relative amounts of hrp36 and snRNPs are compared at particular loci in different preparations either by standard (Fig. 9) or confocal (Fig. 11) microscopy. In both cases, the anti-snRNP antibody strongly stains the 1A locus at the distal tip of the X chromosome while there is little or no anti-hrp36 staining at this site (1A, Figs. 9 and 11). In contrast, there is relatively more hrp36 than snRNPs at the 2A locus in both cases (2A, Figs. 9 and 11). Therefore we believe that the results accurately reflect the distribution of these components on nascent transcripts.

Discussion

We have simultaneously visualized individual hnRNP proteins as they associate with nascent transcripts on *D. melanogaster* polytene chromosomes. Our observations provide direct evidence that the relative amounts of different hnRNP proteins on nascent transcripts are not fixed, but vary, suggesting that the associations of hnRNP proteins with pre-mRNAs are transcript-dependent. Previous studies of hnRNP complexes have led to models predicting that hnRNP particles with a fixed protein stoichiometry nonspecifically bind to a defined length of RNA at intervals along the transcript in a 'nucleosome-like' manner (Samarina et al., 1968; LeStourgeon et al., 1981; Chung and Wooley, 1986; Conway et al., 1988; Barnett et al., 1991). In contrast to these models, our data indicate that hnRNP proteins are not restricted to form complexes containing a fixed ratio of proteins. Rather than generically packaging all transcripts in a uniform manner, our observations suggest that there is a unique arrangement of hnRNP proteins on different transcripts, and this could specifically influence the fates of individual pre-mRNAs. The formation of transcript-specific hnRNP complexes has also been observed in vitro. Complexes formed on several different pre-mRNAs in Hela nuclear extracts before spliceosome formation were isolated and found to consist primarily of hnRNP proteins. Notably, the protein composition of each different transcript was unique, most of the differences being in the relative amounts of individual hnRNP proteins bound (Bennett et al., 1992). Studies with amphibian oocyte lampbrush chromosomes also argued for the formation of hnRNA-specific complexes. At least one hnRNP protein, hnRNP L, preferentially binds to the nascent transcripts of the giant loops of amphibian oocyte lampbrush chromosomes although it also binds, in lower concentrations, most transcripts from the typical loops (Piñol-Roma et al., 1989). Thus, vertebrate and invertebrate hnRNP proteins are likely to assemble with pre-mRNA in
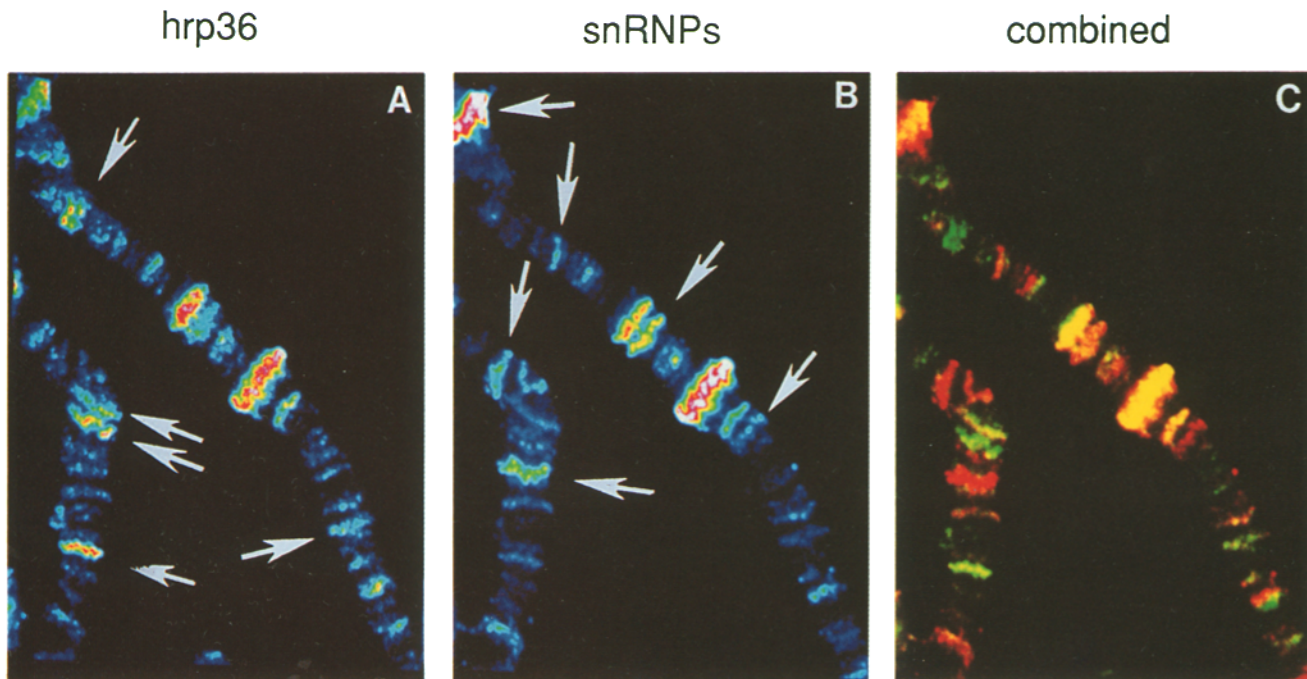
Figure 10. Simultaneous localization of hrp36 and snRNPs on nascent transcripts. Salivary gland polytene chromosomes were stained with monoclonal anti-hrp36 antibodies directly labeled with Texas red (*hrp36, A*) and the monoclonal anti-snRNP antibody Y12, which was directly labeled with FITC (*snRNPs, B*). Loci displaying relative differences in the intensity of staining with these two antibodies are denoted with arrowheads. The confocal micrographs are depicted in the color scheme described in the legend for Fig. 5. In *C*, the anti-hrp36 (*green*) and anti-snRNP (*red*) signals in this field have been merged.
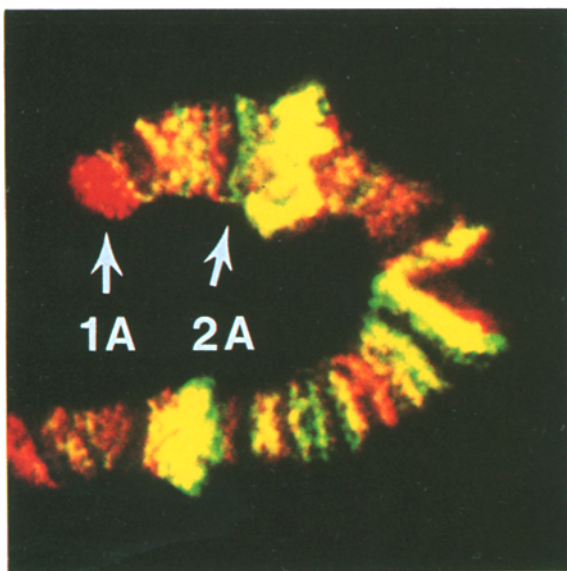
hrp36 and snRNPs

Figure 11. Simultaneous localization of hrp36 and snRNPs on nascent transcripts. Salivary gland polytene chromosomes were stained with monoclonal anti-hrp36 antibodies which were directly labeled with Texas red and the monoclonal anti-snRNP antibody Y12, which was directly labeled with FITC. The anti-hrp36 (*green*) and anti-snRNP (*red*) signals in this field have been merged, and the 1A and 2A loci which contain relatively more snRNPs and hrp36, respectively, are denoted with arrowheads.

a transcript-specific manner. In *D. melanogaster*, pronounced differences in the relative amounts of these proteins on nascent transcripts are apparent even for the abundant and highly related hrp proteins.

The differential association of individual hnRNP proteins with nascent transcripts could be due to differences in the RNA-binding preferences of the individual hnRNP proteins, and also to differences in protein–protein interactions. Although little is known about the protein–protein interactions between RNA-binding proteins, several recent studies indicate that many (if not all) of the hnRNP proteins can preferentially and selectively bind specific RNA sequences in vitro (Moore et al., 1988; Swanson and Dreyfuss, 1988*a,b*; Wilusz et al., 1988; Buvoli et al., 1990). In addition to the intrinsic RNA-binding preferences of individual hnRNP proteins, their binding to pre-mRNAs could be influenced by other factors, including several pre-mRNA–binding proteins which are involved in the regulation of alternative pre-mRNA splicing (for review see Rio, 1992) and snRNPs.

In addition to the differences seen in the relative amounts of individual hnRNP proteins bound to nascent transcripts, we find multiple instances in which hnRNP proteins and snRNP particles associate differentially with nascent transcripts. Thus, it is likely that hnRNP proteins and snRNPs also bind pre-mRNAs independently. This differs from the recently proposed model that hnRNP proteins and snRNP particles associate together in a preassembled unitary particle, which subsequently binds to nascent transcripts in a uniform manner (Wu et al., 1991). We note, however, that as both hnRNP proteins and snRNPs colocalize on many tran-

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scripts, both in previous ultrastructural studies (Fakan et al., 1984, 1986) and in our studies here, it is likely that they interact and influence each other's binding. The U1 snRNP, for example, has been shown to enhance the photochemical crosslinking of hnRNP A1 to pre-mRNA in vitro (Mayrand and Pederson, 1990). Both hnRNP proteins and snRNPs associate with the majority of nascent transcripts, including those that do not contain introns (Sass and Pederson, 1984; Wu et al., 1991 and references therein; Matunis et al., 1992a; Bennett et al., 1992). For example, the intronless hsp70 pre-mRNAs are bound by hnRNPs (Fig. 8) and also by snRNPs (Matunis, E., and G. Dreyfuss, unpublished observations; Martin et al., 1987). It is therefore likely that hnRNP proteins and snRNPs can bind not only to high-affinity binding sites but also to relatively low-affinity, less-specific binding sites. This binding probably has important functions such as presenting the large pre-mRNAs such that they can be processed more efficiently. The subset of these proteins which bind to more specific high-affinity binding sites can form hnRNA–hnRNP–snRNP complexes of specialized function, one example of which is the spliceosome. Since both hnRNP proteins and snRNPs are implicated in several additional aspects of mRNA biogenesis such as poly-adenylation (Moore and Sharp, 1984; Hashimoto and Steitz, 1986) and mRNA transport (Piñol-Roma and Dreyfuss, 1992), numerous types of hnRNA–hnRNP–snRNP complexes are likely to exist on nascent transcripts. While this manuscript was in preparation, Amero et al. (1992) reported a differential association of hnRNP proteins and snRNPs with polytene chromosomes of *D. melanogaster*. However, since they used only one polyclonal antibody to hrp proteins, they were not able to observe the differential association of hnRNP proteins with nascent transcripts.

In summary, the relative amounts of individual hnRNP proteins and snRNP particles on nascent pre-mRNA are not fixed, but vary, and each transcript may have a distinct assembly of these components. It is likely that the specific constellation of hnRNP proteins and snRNP particles on a particular nascent transcript determines the fate of this pre-mRNA. Consistent with this, hnRNP A1, one of the abundant mammalian 2×RBD-Gly proteins, has been recently shown to influence 5' splice site selection and affect the alternative splicing of pre-mRNAs in vitro (Mayeda and Krainer, 1992). Notably, it is the relative amount of hnRNP A1 to that of another pre-mRNA–binding protein, ASF, which determines splice-site selection during pre-mRNA processing. Thus, the specific composition and arrangement of pre-mRNA–binding proteins on a particular RNA is likely to be of major importance to its fate and processing.

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