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Independent deposition of heterogeneous nuclear ribonucleoproteins and small nuclear ribonucleoprotein particles at sites of transcription

(mRNA splicing/RNA processing)

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ABSTRACT **The major nuclear ribonucleoproteins (RNPs) involved in pre-mRNA processing are classified in broad terms either as small nuclear RNPs (snRNPs), which are major participants in the splicing reaction, or heterogeneous nuclear RNPs (hnRNPs), which traditionally have been thought to function in general pre-mRNA packaging. We obtained antibodies that recognize these two classes of RNP in *Drosophila melanogaster*. Using a sequential immunostaining technique to compare directly the distribution of these RNPs on *Drosophila* polytene chromosomes, we found that the two patterns were very similar qualitatively but not quantitatively, arguing for the independent deposition of the two RNP types and supporting a role for hnRNP proteins, but not snRNPs, in general transcript packaging.**

Both heterogeneous nuclear ribonucleoproteins (hnRNPs; reviewed in refs. 1 and 2) and small nuclear ribonucleoproteins (snRNPs; reviewed in ref. 3) are deposited cotranscriptionally on eukaryotic RNA polymerase II transcripts (4–8). Whereas the major basic hnRNP proteins have been considered traditionally to function in general pre-mRNA packaging (2, 9), they have been proposed recently to be specific splicing cofactors or to be preferentially associated with splice junction sequences (10–15). snRNPs are major participants in the splicing reaction (3) but have been implicated recently in general packaging as part of a previously assembled unitary processing complex also containing hnRNPs (5, 6). The various proposals predict different amounts and ratios of the two protein types on nuclear pre-mRNA molecules at chromosomal sites of transcription, which is the issue we have addressed by sequential immunostaining.

The core hnRNP proteins (A, B, and C proteins of 32–45 kDa) were originally identified as the major proteins that are associated with newly synthesized pre-mRNA (in the form of 30–50S RNP particles) when it is extracted from nuclei (reviewed in refs. 1 and 2). This observation, together with their nuclear abundance, their ability to bind single-stranded nucleic acids regardless of sequence, and their helix-destabilizing properties, led to the notion that these core hnRNP proteins are involved in general pre-mRNA packaging, much as the histones are involved in the general packaging of DNA (1, 2). However, more recent investigations of hnRNP proteins, using *in vitro* splicing or *in vitro* RNA binding studies, have suggested that these proteins play a role in the splicing reaction (10–12), that they bind with high affinity to sequences at 3′ splice sites (13, 14), and that they are dependent on snRNPs for acquisition of a crosslinkable association with RNA (13). These *in vitro* studies have led to a reappraisal of the independent structural role of hnRNP proteins in pre-mRNA packaging towards a view that they are a few of the many required cofactors for splicing. The simplest version of this view would predict a constant stoichiometry of snRNPs and the core hnRNP proteins on pre-mRNA, in amounts that correlate with the number of splicing signals.

Another recently proposed model would also predict a constant stoichiometry of snRNPs and hnRNP proteins on pre-mRNA, but in amounts that correlate with RNA length rather than with splicing signals (5, 6). The unitary processing complex proposal (5, 6) predicts codeposition and constant stoichiometry of hnRNP proteins and snRNPs on transcripts and is based on cytological observations of oocyte contents of the newt *Notophthalmus viridescens*. First, hnRNP proteins and snRNPs (plus other splicing factors) occur in the same nuclear extrachromosomal complexes, the B snurposomes, and second, these same components occur on almost all lampbrush chromosome loops in amounts that correlate with RNA mass distribution on that loop (5). Although reports of snRNPs at loci thought not to have introns [*Chironomus* polytene chromosome Balbiani rings (4) and newt histone gene-containing lampbrush loops (5)] appear to support this model, it is now known that the Balbiani ring genes contain introns (16, 17), and splicing signals may occur on extremely long (hundreds of kilobases) readthrough transcription units on lampbrush chromosomes (18).

The original views that the major hnRNP proteins associate promiscuously with pre-mRNA, while snRNPs are deposited specifically at splice sites, are supported by many *in vitro* RNA binding studies (e.g., refs. 8 and 19; reviewed in refs. 1–3), by analysis of RNA sequences associated with extracted hnRNP complexes (reviewed in ref. 1), and by electron microscopic visualization of active genes (20, 21). Thus the abundance of snRNPs at a given transcriptionally active site would reflect the number of introns and the strength of their splicing signals, whereas the abundance of hnRNP proteins would be a function of RNA length, leading to site-specific ratios of hnRNP proteins to snRNPs. Our observations of such site-specific ratios and of intense hnRNP staining at the highly transcribed puff sites support these original predictions.

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Abbreviations: hnRNP, heterogeneous nuclear ribonucleoprotein; snRNP, small nuclear ribonucleoprotein.

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MATERIALS AND METHODS

Antibodies. Four antibodies were used, all of which have been described previously. The anti-*Drosophila* hnRNP antibody was a rabbit antiserum specific for the major basic A/B-type hnRNP proteins (22). The other three antibodies were mouse monoclonal antibodies: 4G3, specific for the mammalian U2 snRNP B″ protein of 28 kDa (23); anti-m_3G cap antibody, which recognizes the snRNA-specific cap (24); and Y1D2, specific for the *Drosophila* nuclear protein PEP, which is found primarily on ecdysone-regulated puffs (25).

Immunoblotting. Nuclear extract was prepared as described (26) from logarithmic-phase *Drosophila* Schneider 2 cells. The nuclear extract was fractionated in SDS/12% polyacrylamide gels, and the separated proteins were either stained with Coomassie blue or electroblotted onto nitrocellulose and probed with undiluted culture supernatant from hybridoma cells secreting antibody 4G3. The primary antibody was detected by using biotinylated horse anti-mouse IgG and the VectaStain ABC immunoperoxidase detection system (Vector Laboratories), according to the manufacturer's instructions.

Immunoprecipitation. Immunoprecipitation experiments utilized protein A-agarose beads (BRL) incubated with rabbit anti-mouse IgG (Zymed) and undiluted hybridoma cell supernatants. The loaded beads were incubated in *Drosophila* Kc cell nuclear extract (26) at 4°C overnight, and washed with 10 mM Tris, pH 7.5/500 mM NaCl/0.05% Nonidet P-40 (4G3 antibody) or 20 mM Hepes, pH 8.0/150 mM NaCl/0.05% Triton X-100 (anti-m_3G antibody) prior to phenol extraction. Total nuclear RNA samples were prepared by phenol extraction of the Kc cell nuclear extract. The RNA samples (either total or immunoprecipitated) were fractionated in 10% polyacrylamide denaturing gels and visualized by silver staining.

Immunofluorescence on Polytene Chromosomes. Immunofluorescence assays on polytene chromosomes from third-instar *D. melanogaster* larvae were performed as described (25, 27), using a 45% acetic acid/3.7% formaldehyde solution to fix the chromosomes. All antibodies were incubated for 2 hr at room temperature on the slides containing the squashed polytene chromosomes. The mouse monoclonal antibodies were detected with a fluoresceinated goat anti-mouse antibody (ICN; 1:1000 dilution), and the rabbit anti-hnRNP antibodies were detected with a goat anti-rabbit antibody conjugated to rhodamine (ICN; 1:500 dilution). Thus, when the same chromosome set was stained with two different antibodies (one raised in mice and one raised in rabbits), the secondary detection systems were noncrossreactive and nonoverlapping. The localization of the first antibody on the chromosomes was recorded by photography using UV illumination appropriate for either rhodamine or fluorescein. The coverslip then was removed from the slide containing the chromosome squash and the slide was washed three times for 5 min, with agitation, in 50 mM Tris, pH 7.6/725 mM NaCl at room temperature. The antibody staining procedure was repeated, as was photography with different UV illumination. For each experiment, the secondary antibodies were shown to produce background levels of fluorescence in the absence of the primary antibody.

RESULTS

As a probe for the deposition of hnRNP proteins, we used an antiserum raised against the major basic A/B-type hnRNP "HRB" proteins from *Drosophila* (22, 28, 29). These 38- to 41-kDa basic proteins are coisolated with *Drosophila* nuclear poly(A)$^+$ RNA and share all properties tested, including sequence similarity and single-stranded nucleic acid-binding properties, with mammalian A/B-type hnRNP proteins (22). This antibody also recognizes HeLa cell A/B-hnRNP proteins on immunoblots (22).

For a snRNP protein probe, we used the monoclonal antibody 4G3 (23). In experiments with mammalian cell nuclear extracts, this antibody recognizes only the U2 snRNP B″ protein of 28 kDa on immunoblots, and immunoprecipitates only U2 snRNP (23). However, this antibody recognized two *Drosophila* nuclear proteins of 36 and 28 kDa on immunoblots (Fig. 1*a*, lane 2) and precipitated two *Drosophila* RNA species (Fig. 1*b*, lane 3) that were also precipitated by the antibody specific to the snRNA-specific 2,2′,7-trimethylguanosine (m_3G) cap (Fig. 1*b*, lane 2). The evidence that these two m_3G-capped RNA species represent *Drosophila* U1 and U2 snRNAs is the following: their size [as expected (30), approximately 190 and 165 nucleotides] based on electrophoretic migration relative to known HeLa cell snRNAs (data not shown), their position of migration relative to endogenous 5S RNA and tRNA (Fig. 1*b*) and mammalian cell snRNAs (see ref. 31), and their characteristic appearance as the two largest abundant and snRNP antibody-precipitable nuclear RNA species in this region of 10% polyacrylamide denaturing gels (Fig. 1*b*; e.g., compare figure 1 of ref. 31). Thus, this antibody, which recognizes only the U2 snRNP B″ protein in mammalian cells, apparently also recognizes a U1 snRNP protein in *Drosophila*. We propose that this second protein is the fly U1 snRNP A protein, based on the ≈80% sequence identity (32) plus antigenic relatedness (23, 33) between the human U1 snRNP A protein (32 kDa) and the human U2 snRNP B″ protein (28 kDa). Thus, the major reactive *Drosophila* protein species on the immunoblot (Fig. 1*a*, lane 2) of 28 kDa would represent the fly U2 snRNP B″ protein, whereas the 36-kDa species would represent the fly

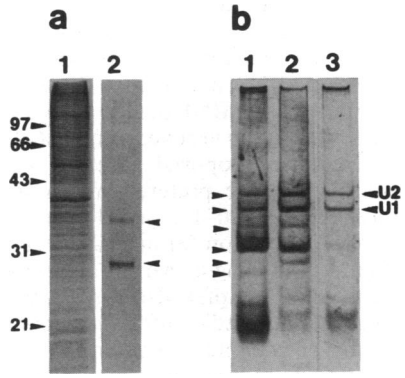

FIG. 1. Recognition of *Drosophila* proteins and snRNPs by the anti-B″ human snRNP protein antibody. (*a*) Schneider 2 cell nuclear extract was fractionated in SDS/12% polyacrylamide gels, which were either stained with Coomassie blue (lane 1) or electroblotted onto nitrocellulose and probed with undiluted cell supernatant from hybridoma cells secreting antibody 4G3 (lane 2). Two bands of 36 and 28 kDa, tentatively identified as *Drosophila* A and B″ snRNP proteins (see text), are recognized. (*b*) RNA samples were prepared from Kc cell nuclear extract either by phenol extraction (lane 1), by immunoprecipitation with anti-m_3G cap antibody (lane 2), or by immunoprecipitation with anti-human B″ protein antibody 4G3 (lane 3). The samples were fractionated in 10% polyacrylamide denaturing gels and visualized by silver staining. The top two arrowheads to the left of lane 1 point to RNA species identified as U2 and U1 snRNA by comparison with migration of mammalian snRNAs (not shown) and with previously reported electrophoretic migrations of *Drosophila* snRNAs (e.g., 30, 31). The U2 and U1 assignments are firm based on their size, abundance, and reproducible relative migration. The remaining arrowheads identify tentatively the small RNA species U4, 5S, U5, and U6 (in descending order); these latter assignments are not directly relevant to the results reported here. Two very abundant cellular RNA species, 5S RNA (labeled as indicated above) and the tRNA population (large smear at the bottom of the gel), are typical contaminants of these nuclear RNA preparations.

U1 snRNP A protein. This antibody thus is well suited as a probe for snRNP deposition, since U1 and U2 snRNPs are known to participate in the early stages of splice site recognition (3).

We devised a sequential indirect immunofluorescent staining procedure with nonoverlapping detection systems to compare directly the distributions of the snRNP proteins (Fig. 2*a*) and the hnRNP proteins (Fig. 2*b*) on the same set of polytene chromosomes. Both patterns were similar to the typical polymerase II transcription pattern (e.g., ref. 25), with staining of visible puffs and interband regions but no staining of extrachromosomal (E) or nucleolar (Nu) debris (see Fig. 2*c*). Staining with an anti-m_3G cap monoclonal antibody (24) (Fig. 2 *f* and *g*) produced a similar puff and interband staining, as expected if the snRNP protein pattern indeed represents the distribution of intact snRNP particles. The specificity of the immunofluorescence assay was shown by the more limited distribution pattern of another nuclear protein, PEP, which was found primarily on the active, ecdysone-regulated puffs (ref. 25; Fig. 2 *d* and *e*). We note also that snRNPs and hnRNPs were distributed differently in an intact polytene nucleus (N in Fig. 2 *a–c*). Although both RNPs occurred in non-nucleolar regions of the intact nucleus, which is largely occupied by polytene chromosomes, the patterns were different in specifics, such as the fine speckling of the hnRNPs (Fig. 2*b*). It is not clear whether the patterns noted here are

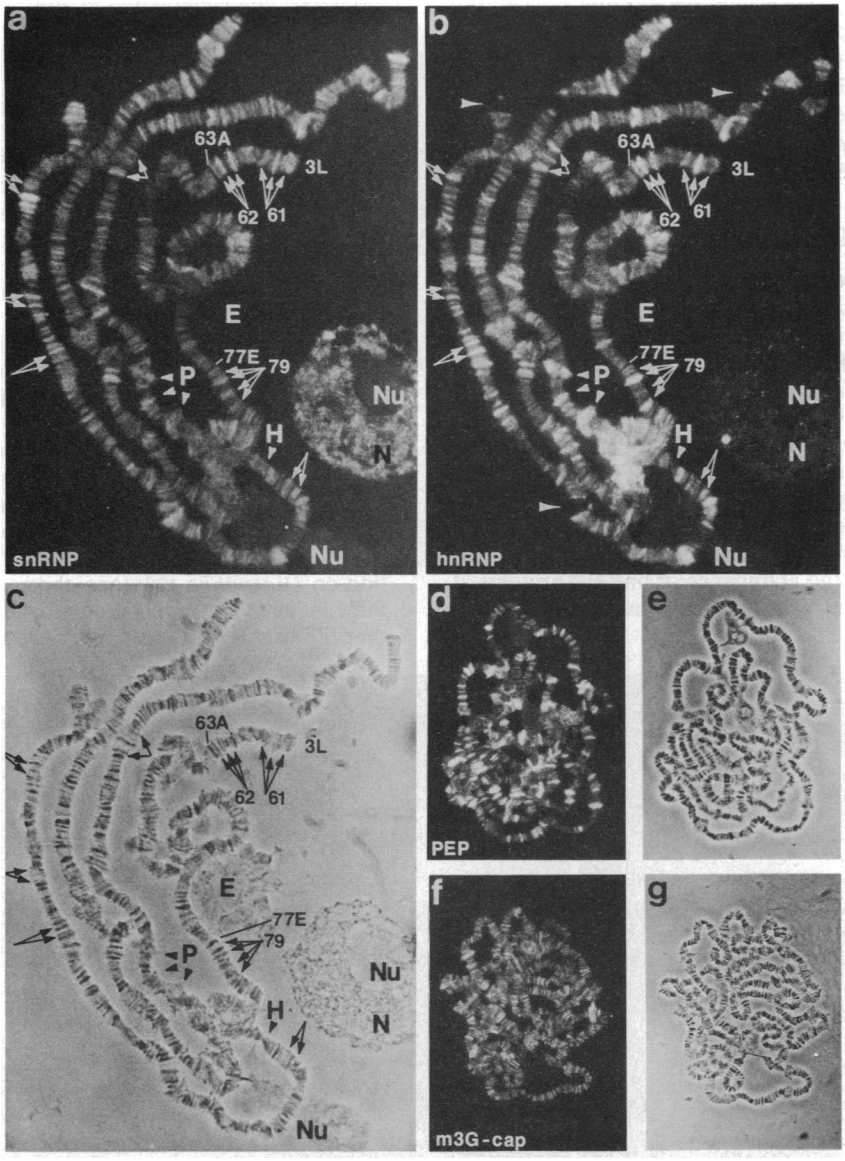

FIG. 2. Sequential staining of polytene chromosomes with snRNP- and hnRNP-specific antibodies. The chromosome set in *a–c* was first stained with the anti-snRNP 4G3 antibody, which was detected with a fluoresceinated goat anti-mouse antibody and then photographed under phase-contrast (*c*) and UV illumination (*a*). The staining procedure was repeated with affinity-purified anti-hnRNP antiserum, which was detected with a goat anti-rabbit antibody conjugated to rhodamine (*b*). Photography was repeated with different UV illumination. Pairs of arrows indicate representative nearby chromosomal sites that display very different staining ratios with hnRNP and snRNP antibodies. Groups of three or four arrows indicate representative specific loci (also identified in Fig. 3), which exhibit the same antibody-specific staining levels regardless of the order of antibody staining (cf. this figure with Fig. 3). Proceeding from the telomere to the chromocenter on the left arm of the third chromosome (3L), these loci fall within the standard polytene chromosome map positions 61A–61F (labeled 61), 62B–62F (labeled 62), and 78A–79D (labeled 79). The bands at 63A and 77E are shown as landmarks. E, extrachromosomal debris; H, histone locus at 39DE; N, nucleus; Nu, nucleolus; P, representative puffs. In *b*, small regions of several chromosomes were lifted off the slide when the cover slip was removed prior to the second staining (unlabeled arrowheads). Also shown: UV illumination (*d*) or phase-contrast (*e*) micrograph of chromosomes stained with monoclonal antibody Y1D2, specific for PEP (25); UV illumination (*f*) or phase-contrast (*g*) micrograph of chromosomes stained with monoclonal antibody against the m_3G-cap specific to snRNPs (24).

unique to the polytene nucleus or are a result of fixation conditions for chromosome squashing (see refs. 34 and 35).

In comparing the snRNP and hnRNP patterns on polytene chromosomes (Fig. 2 *a* and *b*), which essentially were identical qualitatively, numerous quantitative differences could be seen. These were particularly noticeable as differences in snRNP/hnRNP signal ratios at neighboring sites (paired arrows, Fig. 2 *a–c*) and were reproducible on other chromosome sets from the same larva (data not shown). Similar quantitative differences were seen when the order of antibody staining was reversed (Fig. 3). The reproducibility of the staining patterns with the two antibodies was shown by mapping specific representative loci (labeled 61, 62, and 79 in Figs. 2 and 3; see Fig. 2 legend) on the left arm of the third chromosome. They were found to have the same relative staining levels with the two antibodies regardless of the order of antibody staining. With both staining regimens, the hnRNP proteins were abundant in puffs (P in Figs. 2*b* and 3*a*), whereas the snRNP proteins sometimes were abundant in puffs but frequently were not (Figs. 2*a* and 3*b*).

DISCUSSION

In this study, we tested several proposed models for the involvement of snRNPs and hnRNP proteins in the packaging and processing of pre-mRNAs. Our data indicate that snRNPs and major hnRNP proteins are deposited independently on nascent RNAs. The data do not support models in which snRNPs and hnRNP proteins are codeposited as part of a unitary processing/packaging complex (5, 6). The suggestion from *in vitro* studies (10–15) that hnRNP proteins may be localized preferentially at splice sites for a specific function in the splicing reaction is not supported by our observation that hnRNP protein levels do not correlate with snRNP levels. Finally, the abundance of hnRNP proteins at puffed sites is consistent with a general packaging function for these proteins.

The preferential amplification of the hnRNP signal in puffs correlates with RNA mass, since puff size is known to increase as a function of both transcript length and promoter strength (36). Because this anti-hnRNP antibody recognizes a family of approximately nine similar basic proteins (22), which are encoded by two different genes (28, 29), we could not distinguish whether individual A/B-type hnRNP proteins have sequence preferences *in vivo* as they do *in vitro* (14, 15), but we could conclude that the total amount of these proteins at a given site correlates roughly with transcriptional activity. Furthermore, in another recent study, when monoclonal antibodies to three different *Drosophila* A/B-type hnRNP proteins were localized on polytene chromosomes, all three exhibited intense staining of puffed sites, with no obvious protein-specific localization (37).

The snRNP protein (Fig. 2*a*) and snRNA (Fig. 2*f*) signals were not amplified in many puffs but are not necessarily expected to be amplified to the levels observed for hnRNP proteins if snRNPs are binding only to splicing signals. The bulk of the RNA mass in long transcripts (which would be expected in most puffs) is contributed by long introns; the number of snRNP binding sites (splice junctions) would increase less dramatically than the number of hnRNP protein binding sites, assuming that hnRNP proteins bind RNA in a nonspecific and stoichiometric fashion (1, 2, 9, 38). Additionally, snRNP binding efficiency on *nascent* transcripts may vary with the strength of individual splicing signals. Examination of the histone (intronless) gene locus (H, Fig. 2 *a–c*) was not informative in assessing whether the snRNP signal represented splice-site binding, since it was not stained above background levels by either antibody, presumably due to the cessation of DNA synthesis, and thus histone mRNA synthesis, in these late-stage polytene chromosomes.

Our results indicate that hnRNP proteins and snRNPs are not present in stoichiometric amounts at all sites of transcription on *Drosophila* polytene chromosomes, consistent with their independent deposition. An alternative explanation is that the two RNP types are codeposited but are then inde-

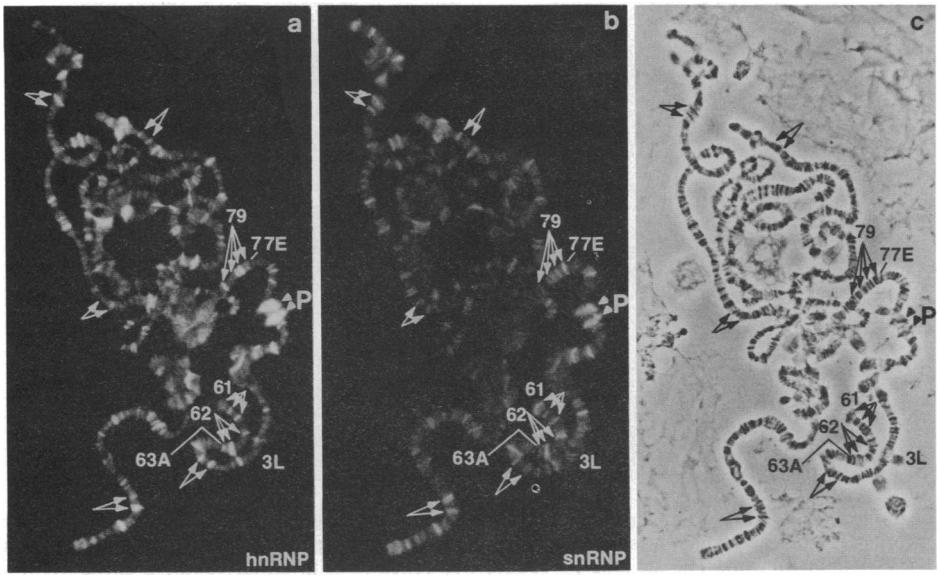

FIG. 3. Sequential staining of polytene chromosomes with hnRNP- and snRNP-specific antibodies. Chromosome staining was as in Fig. 2, except that the order of antibody staining was reversed: anti-hnRNP was used first (*a*), followed by anti-snRNP (4G3) (*b*). Phase-contrast view of same chromosome set is shown in *c*. Pairs of arrows indicate representative nearby chromosomal sites that display very different staining ratios with the two antibodies. P, representative puffs. Specific representative loci have been mapped on the left arm of the third chromosome (see Fig. 2 legend), showing the reproducibility of staining with the two antibodies, regardless of the order of staining. Note that the chromosomes shown here and in Fig. 2 are from somewhat different larval developmental stages, as indicated by the two large puffs (P) at 74E to 75B on 3L that are active on the set shown in this figure, but not on the set shown in Fig. 2. There are numerous other differences in the transcriptional status of specific sites (as indicated by staining with the hnRNP antibody, which is essentially identical to polymerase II antibody staining; S.A.A., unpublished work), including an extra site in the 79 cluster in this figure as compared with the same region in Fig. 2.

pendently susceptible to dissociation. We favor the former, simpler explanation because it is consistent with electron microscopic observations in which the earliest RNA packaging steps were visualized on nascent RNA and evidence for specific particle deposition was seen only at splice sites (20, 21). If snRNPs are indeed codeposited with hnRNP proteins on amphibian oocyte lampbrush chromosome loops (refs. 5 and 6; see Introduction), this phenomenon does not extend to *Drosophila*. As shown here at the gene population level and as supported previously by observations of individual genes at the electron microscopic level (20, 21), RNP abundance reflects RNA mass whereas snRNP abundance probably reflects early splicing activity at an active locus. The amount of hnRNP protein seen is not that predicted by reports suggesting the "specific" association of major hnRNP proteins with 3′ splice sites (14, 15) or the "more stable" association of those hnRNPs in the vicinity of snRNPs (13). These *in vitro* studies predict a positive correlation between the abundance of stable hnRNPs and snRNPs if these "high-affinity" binding sites are the only targets for their deposition. However, the nuclear abundance of hnRNP proteins, which ensures their excess over these high-affinity sites, and the known RNA-binding and helix-destabilizing properties of these proteins (1, 2, 9, 38) argue for binding of hnRNPs along the entire transcript length *in vivo*. Although their binding may be nonspecific in terms of sequence recognition, the role that they play in managing long transcripts is probably very important. If left naked, RNA will not only be accessible to nuclease attack but will also adopt complex higher-order structures that have a high probability of masking the short consensus sequences recognized by specific RNA-processing factors. By binding to the nascent transcript within 100 nucleotides or less of its emergence from the polymerase complex (39), these helix-destabilizing proteins presumably facilitate the very rapid, cotranscriptional splicing observed *in vivo* (e.g., refs. 21 and 40). Moreover, a role in general packaging of pre-mRNAs does not preclude a specific splicing function for core hnRNP proteins, as suggested by a recent *in vitro* splicing study in which specific effects were observed when hnRNP A1 protein was present in stoichiometric excess over the pre-mRNA substrate (12). One can envision, in fact, that specific effects of hnRNP proteins on splicing may be a direct result of their general packaging function; for example, effects of hnRNP protein binding on the kinetics of splice site recognition or on the flexibility properties of nascent transcripts might influence splice-site selection.

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