Introns Excised from the *Delta* Primary Transcript Are Localized Near Sites of *Delta* Transcription

Casey C. Kopczynski and Marc A. T. Muskavitch

Program in Genetics, Cell and Developmental Biology, Department of Biology, Indiana University, Bloomington, Indiana 47405

Abstract. Introns excised from the primary transcript of *Delta* (*Dl*), a *Drosophila* neurogenic gene, accumulate to unusually high levels in embryos. High resolution in situ hybridization reveals a striking localization of the excised introns to two foci per embryonic nucleus. The number of foci can be altered by varying the number of *Dl* genes present in the embryonic nucleus, suggesting that the excised introns are localized near sites of *Dl* transcription. This conclusion is supported by the observation that larval and imaginal disc nuclei containing two copies of *Dl* exhibit only one focus of intron accumulation, as expected for nuclei in which homologous chromosomes are paired. Interestingly, the excised introns do not appear to diffuse away from the foci until late prophase, at which time the foci disperse into numerous small dots of hybridization. These results suggest that the excised *Dl* introns may be associated with a structural element within the nucleus that is dissociated during cell division.

Most genes in higher eukaryotes contain introns that are transcribed into pre-mRNA and subsequently removed by RNA splicing. The ability to reproduce RNA splicing in vitro has permitted a detailed characterization of many of the steps involved in this process (reviewed in Maniatis and Reed, 1987; Sharp, 1987; Guthrie and Patterson, 1988; Ruby and Abelson, 1991). However, little is known about the mechanics of RNA processing as it occurs within the highly structured nucleus. For example, the mechanism by which pre-mRNAs and excised introns are retained in the nucleus while mRNAs are exported is currently unknown. Similarly, the process of snRNP "recycling," which requires the dissociation of snRNPs from excised introns, is poorly understood.

A number of laboratories have characterized the nuclear distribution of components of the splicing machinery to gain insight into the relationship between RNA processing and nuclear structure. Immunofluorescent staining with anti-snRNP antibodies has revealed 20–50 discrete regions within the nucleus where snRNPs are concentrated, as well as low levels of snRNPs in the surrounding nucleoplasm (Lerner et al., 1981; Spector et al., 1983; Reuter et al., 1984; Nyman et al., 1986). Recently, a non-snRNP factor required for spliceosome assembly was shown to be localized specifically to the regions of snRNP concentration, suggesting that these regions may represent active splicing compartments within the nucleus (Fu and Maniatis, 1990). Immunoelectron microscopic analyses further suggest that these putative splicing compartments form a reticular network that extends from the center of the nucleus to the nuclear periphery (Spector et al., 1983; Fakan et al., 1984; Spector, 1990).

Additional evidence for a functional relationship between nuclear structure and RNA processing has come from the localization of specific pre-mRNAs within the nucleus. The nuclei of cells latently infected with Epstein-Barr virus (EBV) accumulate unprocessed EBV RNA along "tracks" that extend from the site of transcription to the nuclear periphery (Lawrence et al., 1989). It has been suggested that these "tracks" may represent specific channels for RNA splicing or mRNA export within the nucleus. In addition, a potential role for the nuclear lamina in RNA processing has been suggested based on the observation that acetylcholine receptor (AChR) intronic sequences are localized at the nuclear periphery in myoblasts (Berman et al., 1990).

Further insight into the spatial organization of the splicing machinery could be obtained by determining where introns accumulate after they have been excised from pre-mRNA. For example, excised introns might accumulate along "tracks" similar to those observed for EBV RNA if splicing occurs during transport to the nuclear periphery. Alternatively, the distribution of excised introns might reveal specific nuclear sites where snRNP "recycling" occurs. To address this issue, we have taken advantage of an unusual feature of the *Drosophila Delta* (*Dl*) gene: introns excised from the *Dl* primary transcript accumulate to levels readily detectable by RNA blot analysis and in situ hybridization (Kopczynski et al., 1988; Kopczynski and Muskavitch, 1989; Haenlin et al., 1990; this report). We find that the accumulation of excised *Dl* introns is highly localized within the nucleus near sites of *Dl* transcription, indicating that splicing of the *Dl* pre-mRNA occurs near the *Dl* gene. Fur-

Casey C. Kopczynski's current address is Department of Molecular and Cell Biology, University of California, Berkeley, California 94720.

thermore, we observe that this localization of excised introns is disrupted during mitosis at late prophase, just before the reported dispersal of many nuclear matrix-associated antigens (reviewed in Verheijen et al., 1988). The implications of these results for models of RNA processing within the nucleus are discussed.

Materials and Methods

Drosophila Stocks

Df(3R)Dl^{12} is described in Alton et al. (1988), *Dp(3;3)bxd*^{110} in Lindsley and Grell (1968), and *TM6C* in Craymer (1984). Tn4b is a second chromosome insertion of a P-element transposon carrying the 24-kb *Dl* transcription unit with 7 kb of 5′-flanking sequence and 2 kb of 3′-flanking sequence (Kopczynski, 1991).

RNA Blot Hybridization

Preparation of total embryonic RNA and oligo(dT)-selection of poly(A)$^+$ RNA were performed as previously described (Kopczynski et al., 1988), except total RNA preparations were further purified by LiCl precipitation (Sambrook et al., 1989). RNA samples were resolved on formaldehyde/agarose gels and subjected to partial alkaline hydrolysis (Maniatis et al., 1982) before transfer to BioTrans nylon membranes (ICN, Costa Mesa, CA). Blot hybridization and ^{32}P labeling of DNA probes were performed as described by Feinberg and Vogelstein (1983). The lengths of the RNA species identified were estimated from their mobility relative to a 0.24–9.5-kb RNA ladder (BRL, Gaithersburg, MD).

Primer Extension Analysis

25 pg of ^{32}P-end-labeled primer was hybridized to 50 μg oligo(dT)-selected RNA and extended with reverse transcriptase as described in Ausubel et al. (1989), except that a mixture of avian myeloblastosis virus (Boehringer Mannheim Biochemicals, Indianapolis, IN) and Moloney murine leukemia virus (BRL) reverse transcriptases was used. The primers used were 3.9-1 (5′-CAAGTTGCGGGTCTGTGCGAGC-3′) and 2.2-1 (5′-GCTATCTACAAGCAGCAAACTAAA-3′).

In Situ Hybridization

RNA probes were internally labeled with digoxygenin-UTP (Boehringer Mannheim Biochemicals) by in vitro transcription according to the manufacturer's instructions. Probes were reduced in length to ~100–300 nucleotides by incubating for 10 min in 1 M sodium carbonate, pH 10.2, at 60°C. Hybridization of the probes to whole, paraformaldehyde-fixed embryos was performed as described in Tautz and Pfeifle (1989), except 5% (wt/vol) dextran sulfate (Pharmacia Fine Chemicals, Piscataway, NJ) was included in the hybridization solution, and the hybridization was performed at 52°C for 12–18 h. Embryos were subsequently washed at 52°C for 6–18 h in five changes of hybridization solution (without dextran sulfate), then for 30 min in PBT (130 mM NaCl, 10 mM sodium phosphate, pH 7.2, 0.1% [vol/vol] Tween 20). To visualize the hybridized probe, embryos were incubated at room temperature for 1.5 h with HRP-conjugated antidigoxygenin (150 U/ml; Boehringer Mannheim Biochemicals) diluted 1:200 in PBT + 5% (vol/vol) normal goat serum, washed for 1.5 h in four changes of PBT, then incubated with 0.5 mg/ml 3,3-DAB, 0.003% H_2O_2 in PBT until the embryos were light brown in color. After three brief washes in PBT, the DAB precipitate was rendered opaque by silver precipitation (Gallyas et al., 1982; Liposits et al., 1984). Embryos were mounted in methyl salicylate for microscopy or stained with 1 μg/ml Hoechst 33342 for 10 min and mounted in 80% (vol/vol) glycerol in H_2O.

The same protocol was used for in situ hybridization of digoxygenin-labeled probes to larval tissues and imaginal discs, except that heptane, required during embryo fixation to permeabilize the vitelline membrane, was usually omitted from the fixation step.

Results

The Minor Dl RNAs Are Excised Introns

Delta function is required for the proper specification of numerous cell fates during *Drosophila* development (Lehmann et al., 1983; Hartenstein and Campos-Ortega, 1986; Vässin and Campos-Ortega, 1987; Shepard et al., 1989; Heitzler and Simpson, 1991). Transcription of the *Dl* gene gives rise to eight distinct RNAs (Vässin et al., 1987; Kopczynski et al., 1988; Kopczynski and Muskavitch, 1989; Haenlin et al., 1990). The two most abundant RNAs, 5.4 and 4.5 kb in length, as well as a less abundant 3.6-kb maternal RNA, encode an 833-amino acid transmembrane protein (Vässin et al., 1987; Kopczynski et al., 1988; Haenlin et al., 1990). The less abundant ("minor") zygotic RNAs are enriched in oligo(dT)-selected RNA preparations (Kopczynski et al., 1988; Haenlin et al., 1990) and sediment as large, EDTA-sensitive ribonucleoproteins in sucrose gradients (Kopczynski and Muskavitch, 1989), two properties characteristic of messenger RNAs. However, the minor RNAs are subcellularly localized in the vicinity of the nucleus (Kopczynski and Muskavitch, 1989), raising the possibility that their accumulation might be restricted to the nucleus.

We have furthered our characterization of the minor RNAs by mapping the genomic positions of DNA fragments that hybridize specifically to the minor RNAs. Fig. 1 *A* compares the minor RNA "hybridization map" to the intron/exon map of the *Dl* mRNAs (Haenlin et al., 1990; Fechtel, K., K. A. Bauer, and M. A. T. Muskavitch, manuscript in preparation). The results show that all of the genomic fragments that hybridize to the minor RNAs are located within *Dl* intronic regions. Most importantly, the length of each of the five minor RNAs estimated from RNA blots is very close to the length of the *Dl* intron with which it hybridizes (Figure 1 *B*). This suggested that the minor RNAs may, in fact, be excised introns. To test this hypothesis, the 5′ ends of the 3.7- and 2.2-kb minor RNAs were mapped by primer extension analysis (Fig. 1 *C*). The 5′ end of the 3.7-kb RNA maps precisely to the exon 3/intron C donor splice site, while the 5′ end of the 2.2-kb RNA maps one nucleotide downstream of the exon 5/intron E donor splice site. We also found that the 5′ ends of the 3.5- and 2.8-kb minor RNAs map to the exon 2/intron B donor splice site and the exon 4/intron D donor splice site, respectively (data not shown). These data strongly support the conclusion that the minor RNAs are introns excised from the *Dl* primary transcript. The alternative possibility, that the minor RNAs are transcribed from independent promoters located at the splice junctions, is highly unlikely since transcription of the minor RNAs and the *Dl* mRNAs requires the same upstream promoter region (Haenlin et al., 1990). The conclusion that the minor RNAs are excised introns is further supported by the restricted accumulation of the minor RNAs within the nucleus (see below).

We estimate the steady state level of each excised intron to be ~5% of *Dl* mRNA levels based upon RNA blot analysis performed using total (unfractionated) embryonic RNA (Fig. 1 *B*, lanes *Dll* and *A3*, and data not shown). Since excised introns are usually rapidly degraded in the nucleus, the accumulation of excised *Dl* introns suggests that these introns have relatively long half-lives.

Excised Dl Introns Are Highly Localized within the Nucleus

We could not determine from our previous in situ hybridization data whether the excised *Dl* introns might be localized to a specific region of the nucleus, as has been reported for

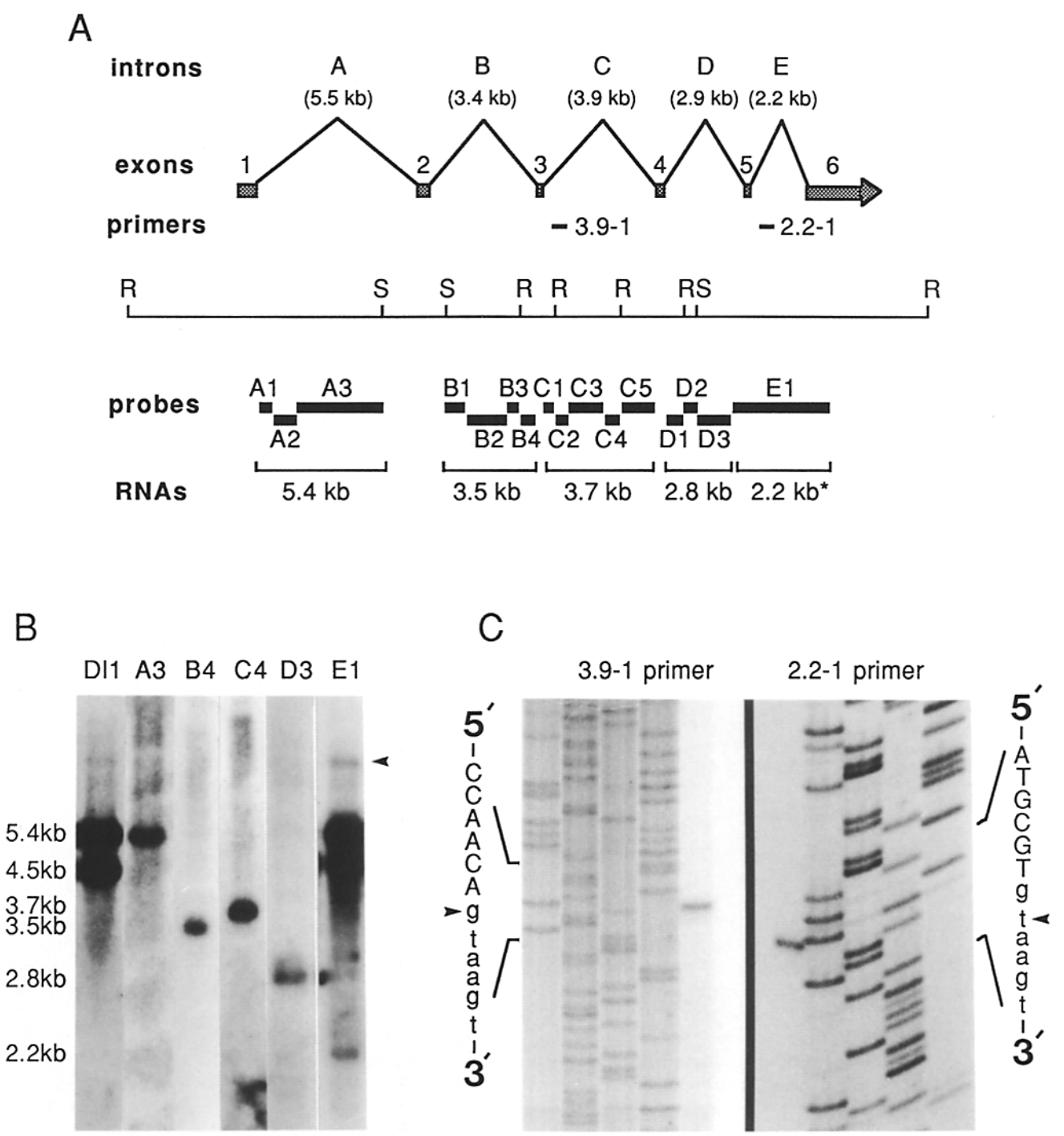

Figure 1. Delta minor RNAs are excised introns. (*A*) Intron/exon map of the *Delta* gene (Haenlin et al., 1990; Fechtel, K., K. A. Bauer, and M. A. T. Muskavitch, manuscript in preparation). The approximate lengths of the *Dl* introns are indicated. The 3.9-1 and 2.2-1 primers are antisense oligonucleotides used in primer extension analyses. Genomic DNA fragments used for RNA blot analyses are represented by bars labeled A1–E1. The lengths of the RNAs detected by the genomic fragments are indicated. (*): fragment E1 also hybridizes to the abundant 5.4- and 4.5-kb RNAs and the minor 2.8-kb RNA. (*B*) RNA blot analysis using intron probes. ^{32}P-labeled DNA probes were hybridized to 20 μg of 3–6 h embryonic total RNA (*Dll*, *A3*) or 10 μg oligo(dT)-selected RNA (*B4–E1*). (*Dll*) *Dl* cDNA (Kopczynski et al., 1988); (*A3*, *B4*, *C4*, *D3*) representative genomic fragments derived from introns A, B, C, and D, respectively; (*E1*) genomic fragment containing sequences from the 2.9-kb intron, 2.2-kb intron, exon 5, and exon 6. The approximate length of each RNA species detected is indicated. The high molecular weight transcript in lane E1 (*arrowhead*) is only observed when probes containing exon sequences are hybridized to oligo(dT)-selected RNA; it is probably an incompletely spliced pre-mRNA. (*C*) Primer extension analysis. Primers 3.9-1 and 2.2-1 were end-labeled with ^{32}P, hybridized to 50 μg oligo(dT)-selected RNA, then extended with reverse transcriptase. The extension products were resolved on gels next to sequencing reactions primed by the same oligonucleotide. The arrowheads indicate the positions of the 3.9-1 and 2.2-1 primer extension products relative to the exon 3/intron C and exon 5/intron E splice site sequences, respectively. (*Uppercase letters*) Exon sequence; (*lowercase letters*) intron sequence. Sequencing reactions were loaded in the order "GATC," from left to right.

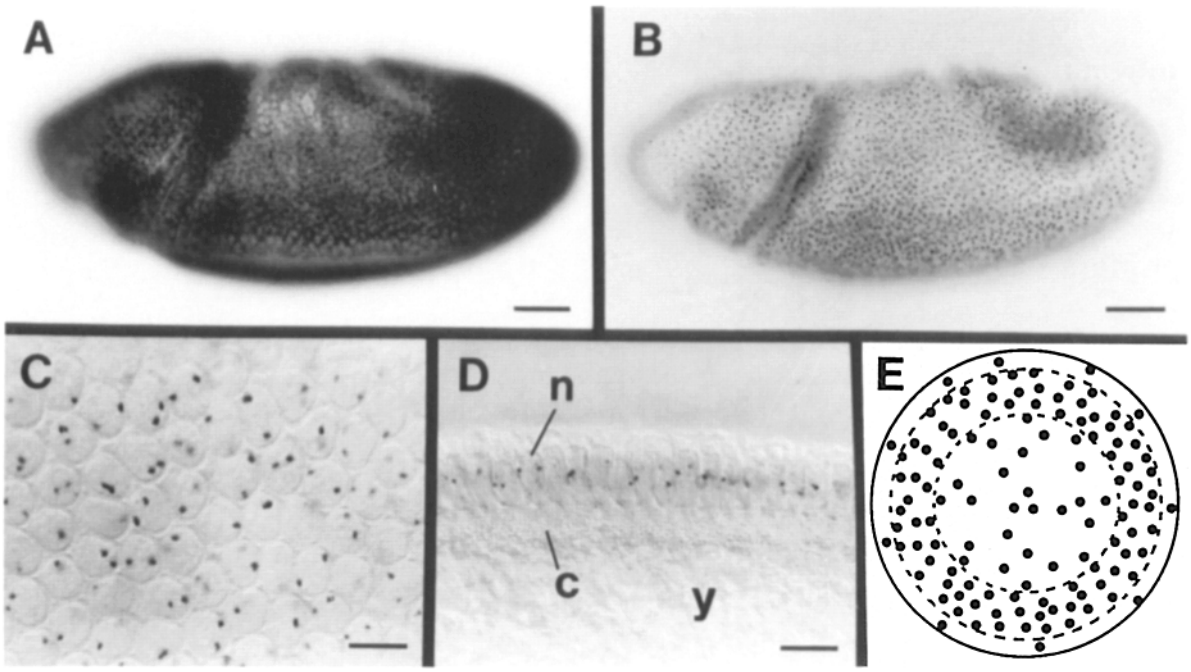

Figure 2. Excised introns accumulate at highly localized foci within embryonic nuclei. Whole embryos were hybridized with digoxygenin-labeled antisense RNA. After washing, the hybridized probe was detected histochemically using a HRP-coupled anti-digoxygenin antibody. The histochemical reaction product was rendered opaque by silver precipitation to increase the sensitivity of the technique. (*A* and *B*) Gastrulation stage embryos hybridized with a D11 cDNA probe (*A*) or an intron-specific probe generated from genomic fragments A2–D3 (Fig. 1 *A*) (*B*). This intron probe has a potential sequence complexity of 12 kb and thus provides an optimal hybridization signal. (*C*) Optical cross section through nuclei of a precellularization stage embryo hybridized with the intron probe. (*D*) Optical sagittal section through nuclei of a cellularization stage embryo hybridized with the intron probe. (*n*) Nucleus; (*c*) cytoplasm; (*y*) yolk. (*E*) Radial distribution of 124 intron accumulation sites from 73 precellularization stage nuclei superimposed on a single nucleus. Each site was placed on the diagram based upon its distance from the nuclear envelope relative to the diameter of the nucleus in which it was located. Measurements were only recorded when the accumulation site and the nuclear membrane were in the same focal plane. The dotted lines delimit a region within which the density of accumulation sites is threefold greater than within the surrounding area. Bars: (*A* and *B*) 50 μm; (*C*) 5 μm; (*D*) 10 μm.

EBV and AChR RNAs. We therefore repeated our analysis using a nonradioactive in situ hybridization protocol (Tautz and Pfeifle, 1989) to achieve greater resolution than previously possible with ^{35}S-labeled probes. We modified the in situ hybridization protocol described by Tautz and Pfeifle (1989) to include a silver precipitation step (Gallyas et al., 1982; Liposits et al., 1984), which renders the normally translucent histochemical reaction product opaque. This modification greatly enhances the sensitivity of the technique.

Fig. 2 shows the different hybridization patterns observed in wild type embryos using a *Dl* cDNA probe and a probe specific for introns A–D. In contrast to the *Dl* mRNA, which accumulates predominantly in the cytoplasm (Fig. 2 *A*), the introns accumulate specifically at two foci within each embryonic nucleus (Fig. 2, *B–D*). Two foci are also observed using a probe that recognizes intron C alone (data not shown), suggesting that all *Dl* introns accumulate in the same two regions within each nucleus. A fraction of the signal observed probably corresponds to incompletely spliced pre-mRNA, since low levels of high molecular weight RNA are detected when intron probes are hybridized to total embryonic RNA (Fig. 1 *B*, lane *A3*). However, the majority of the hybridization signal observed on total RNA blots probed with intron probes corresponds to excised introns (Fig. 1 *B* and data not shown), strongly suggesting that the majority of the signal observed in the nucleus represents hybridization to excised introns. Control hybridizations using sense-strand probes of similar complexity or RNAse-treated embryos revealed no detectable hybridization of the intron probe to *Dl* chromosomal DNA (data not shown).

The excised intron foci often appear to be located near the nuclear envelope (Fig. 2, *C* and *D*). Since it has been suggested that RNA splicing may occur in association with nuclear pores (Blobel, 1985; Urlaub et al., 1989; Berman et al., 1990), we were interested in determining if this localization occurs more frequently than expected for a random distribution of foci. To address this question, the distances between the nuclear membrane and each of 124 foci (from 73 embryonic nuclei) were measured relative to the diameter of the nucleus. These values were then used to superimpose the foci onto a single model nucleus. Fig. 2 *E* shows that the distribution of foci within the embryonic nucleus is not random; the density of foci is threefold higher in the area between the dotted lines than in the surrounding areas. However, it is unlikely that this localization results from an association between RNA splicing and nuclear pores since this region is not adjacent to the nuclear membrane. We also note that

30% of the foci are located outside of this region, indicating that the sites of intron accumulation are not restricted to a specific domain within the nucleus.

Excised Dl Introns Accumulate Near Sites of Transcription

Since excised introns remain associated with spliceosomal snRNPs (Pikielny et al., 1986; Cheng and Abelson, 1987; Konarska and Sharp, 1987), the excised intron foci could represent specific sites of spliceosome disassembly and intron turnover within the nucleus. Alternatively, the excised *Dl* introns might be accumulating at sites of *Dl* transcription, as suggested by the presence of two foci within each nucleus. To distinguish between these possibilities, we tested whether the number of foci observed within the nucleus is dependent upon the number of *Dl* genes present. Fig. 3 shows that embryos heterozygous for a deficiency of *Dl* have only one site of intron accumulation per nucleus (Fig. 3 *A*), whereas embryos heterozygous (Fig. 3 *C*) or homozygous (Fig. 3 *D*) for a duplication of *Dl* ($Dp(3;3)bxd^{110}$; Lindsley and Grell, 1968) have three and four sites of intron accumulation per nucleus, respectively. Thus, the number of foci corresponds precisely to the number of *Dl* genes present in the nucleus, suggesting that the introns accumulate at sites of *Dl* gene transcription.

As an additional test of this hypothesis, the intron probe was hybridized to embryos carrying a *Dl* transposon on the second chromosome. Genetic analyses indicate that the *Dl* gene carried by the transposon is expressed at lower levels than the wild type *Dl* gene (Kopczynski, 1991). Fig. 3 (*E* and *F*) show that embryos carrying one copy of the wild type *Dl* gene and two copies of the *Dl* transposon have one large focus and two smaller foci of hybridization per nucleus. The number and sizes of the foci suggest that they represent the wild type gene and the two *Dl* transposons, respectively. These data strongly support the conclusion that the excised *Dl* introns accumulate near sites of *Dl* gene transcription. We also note that the *Dl* transposons (Fig. 3 *F*, *arrows*) appear to be located at a more basal position within the nucleus than the wild type *Dl* gene (Fig. 3 *E*, *arrows*). This probably reflects the chromosomal positions of the transposon and the wild type *Dl* gene relative to their respective centromeres, since chromosomes in these nuclei are oriented with centromeres positioned apically and telomeres positioned basally (Foe and Alberts, 1985).

Physical Evidence for Pairing of Homologous Chromosomes in Imaginal Nuclei

Homologous chromosomes are physically paired in larval polytene nuclei, and genetic experiments have suggested that they are also paired in imaginal nuclei (Lewis, 1954; Garcia-Bellido and Wandosell, 1978; Jack and Judd, 1979; Gelbart, 1982). If the excised introns are localized at sites of *Dl* transcription, the intron foci should be colocalized in larval and imaginal nuclei since both copies of the *Dl* gene should be immediately apposed within the nucleus. Fig. 4 *A* shows that wild type eye imaginal disc nuclei that express *Dl* contain only one site of intron accumulation, even though two *Dl* genes are present. Similarly, eye imaginal disc nuclei homozygous for a *Dl* duplication (four copies of *Dl*) contain only two sites of intron accumulation (Fig. 4 *B*), as do polytene

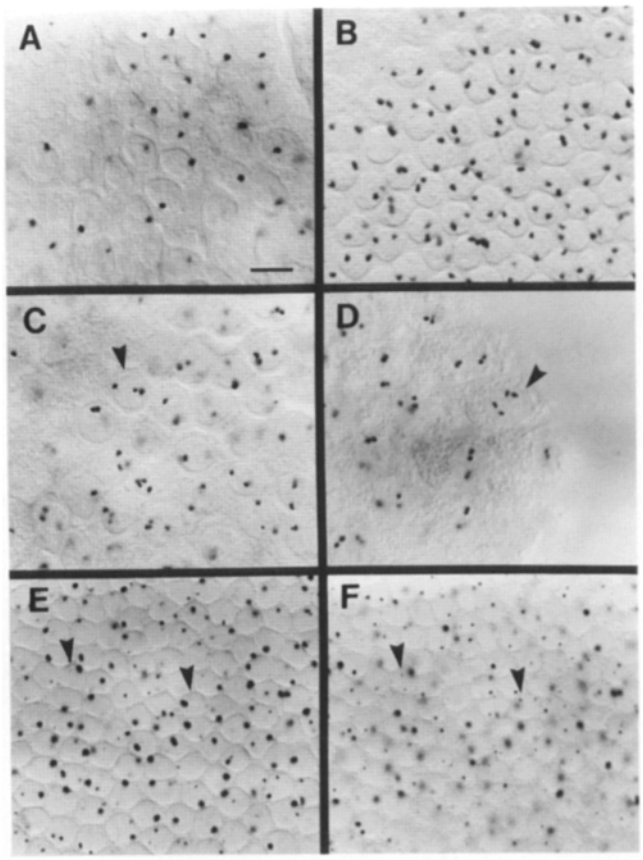

Figure 3. Excised introns are localized near sites of *Dl* transcription. Intron accumulation sites in embryos carrying different numbers of *Dl* genes were visualized by in situ hybridization as described in Fig. 2. Embryos were obtained from the following stocks: $Df(3R)Dl^{12}/TM6C$ (embryos with one or two copies of *Dl*); $Dp(3;3)bxd^{110}/TM6C$ (embryos with three or four copies of *Dl*); or Tn4b;$Df(3R)Dl^{12}/TM6C$ (embryos with two copies of a *Dl* transposon [Tn4b] on the second chromosome and one or two copies of *Dl* on the third chromosome). Embryo genotypes were deduced from the ratio of animals within a collection that exhibited zero, one, two, three, or four foci of intron accumulation. (*A*) Nuclei of a $Df(3R)Dl^{12}/TM6C$ embryo (one copy of *Dl*). A single site of intron accumulation is present. (*B*) Nuclei of a *TM6C/TM6C* embryo (two copies of *Dl*). Two sites of intron accumulation are present. (*C*) Nuclei of a $Dp(3;3)bxd^{110}/TM6C$ embryo (three copies of *Dl*). The arrow points to a nucleus in which the three sites of intron accumulation are in the same focal plane. (*D*) Nuclei of a $Dp(3;3)bxd^{110}/Dp(3;3)bxd^{110}$ embryo (four copies of *Dl*). The arrow points to a nucles in which the four sites of intron accumulation are in the same focal plane. (*E* and *F*) Nuclei of a Tn4b;$Df(3R)Dl^{12}/TM6C$ embryo (three copies of *Dl*). The arrows point to typical nuclei in which the large focus of hybridization is apically localized (*E*) and the two smaller foci are basally localized (*F*). Bar, 5 μm.

nuclei of the larval trachea that contain four copies of *Dl* (Fig. 4 *C*). We have observed that the two sites of intron accumulation in the large polytene nuclei are always relatively close to one another, consistent with the fact that the *Dl* duplication is located on the third chromosome only eight map units away from the wild type *Dl* gene. These data further support the conclusion that the excised *Dl* introns accumulate specifically at sites of *Dl* transcription and repre-

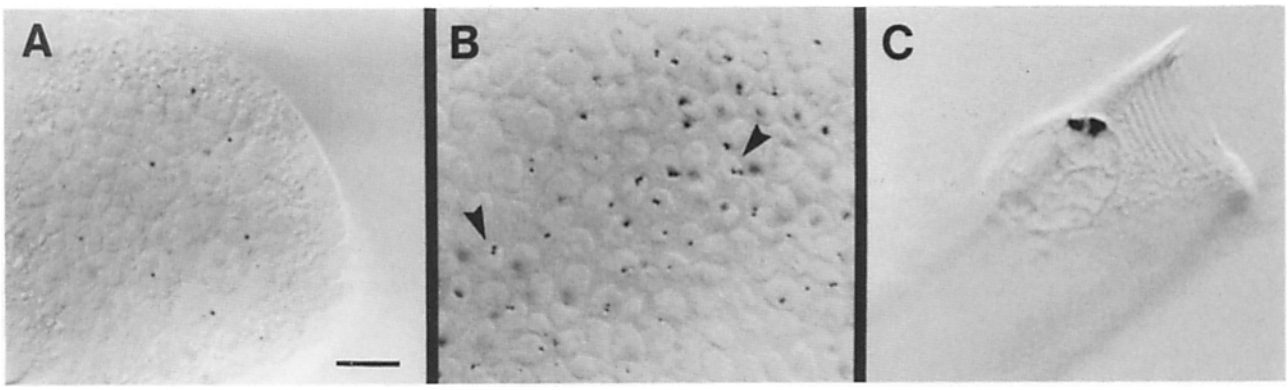

Figure 4. Colocalization of excised intron foci in postembryonic nuclei. Heads dissected from third instar larvae were fixed and hybridized with an intron-specific probe as described in Fig. 2. For microscopy, imaginal discs and trachea were removed from the head and mounted in glycerol. (*A*) Eye disc from a wild type third instar larva (two copies of *Dl*). The nuclei of the peripodial membrane exhibit a single site of intron accumulation. (*B*) Eye disc from a $Dp(3;3)bxd^{110}/Dp(3;3)bxd^{110}$ third instar larva (four copies of *Dl*). Two sites of intron accumulation are present in peripodial membrane nuclei (*arrows*). (*C*) Tracheal segment from a $Dp(3;3)bxd^{110}/Dp(3;3)bxd^{110}$ third instar larva (four copies of *Dl*). Two sites of intron accumulation are present within the large polytene nucleus. Bar, 5 μm.

sent the first physical evidence for homologous pairing of interphase chromosomes in imaginal nuclei.

Mitosis Disrupts Excised Intron Localization

A transient lack of intron hybridization is observed in specific embryonic domains during early germ band elongation (Fig. 5 *A*). The positions of these domains and the timing with which they appear correspond well with the domains of mitotically active cells present during this stage of embryogenesis (Foe, 1989). At higher magnification, it is evident that some nuclei within these domains contain small "dots" of hybridization in addition to the two relatively large foci normally seen during interphase (Fig. 5 *B*). We also observe nuclei in these domains that contain two foci of reduced size and 20–30 "dots" of hybridization, nuclei with two or four small foci and no detectable "dots," and cells without any detectable hybridization (see below).

To determine whether the changes in *Dl* intron distribution are correlated with different stages of mitosis, we used a DNA-specific dye to visualize chromosomes in embryos hybridized with the intron probe. Fig. 5 reveals that during early prophase (Fig. 5, *C* and *D*, *large arrow*), introns remain highly localized within the nucleus. By late prophase (Fig. 5, *C* and *D*, *small arrows*), however, small "dots" of hybridization become apparent in addition to the larger foci. The larger foci appear to be localized with the condensing chromosomes at this stage (Fig. 5, *C* and *D*), while the smaller "dots" are often spatially separate from the fluorescent DNA (data not shown). During metaphase, the small "dots" are no longer detectable (Fig. 5, *E* and *F*, *large arrow*). Instead, two pairs of small foci are visible over the condensed chromosomes. The four small foci present during metaphase are further reduced in size by late anaphase and appear to have segregated with the chromosomes such that two foci are now present at each spindle pole (Fig. 5, *E* and *F*, *small arrow*). These foci may represent nascent pre-mRNA trapped by chromosome condensation, or excised introns that become peripherally associated with the condensed chromosomes. By telophase, hybridization of the intron probe is no longer detectable (Fig. 5, *G* and *H*). These data indicate that the dispersal and apparent degradation of the excised introns is a highly ordered process which, based upon the time required to complete mitosis at this stage (Foe, 1989), is completed in <5 min at 25°C.

Discussion

Nuclear Accumulation of Excised Dl Introns

We have shown that the five minor *Dl* RNAs present in oligo(dT)-selected RNA preparations are excised *Dl* introns. This conclusion is based upon the correlation between the lengths of the introns and the lengths of each of the minor RNAs, the fact that the 5′ ends of the 3.7- and 2.2-kb minor RNAs map to the predicted intron splice junctions, and the accumulation of the minor RNAs within the nucleus. The results of Haenlin et al. (1990), which also revealed that the transcription of all *Dl* RNAs requires the same promoter region, led these authors to similarly conclude that the minor RNAs are excised introns. Since excised introns are not likely to be substrates for polyadenylation, their retention on oligo(dT) columns is probably due to internal adenylate-rich sequences.

We could not determine whether the excised introns are in a lariat or linear conformation because their large sizes prevented the use of PAGE to characterize their structure (Grawbowski et al., 1984; Ruskin et al., 1984). However, a number of investigations have shown that excised introns exist predominantly as lariats in vivo (Domdey et al., 1984; Rodriguez et al., 1984; Zeitlin and Efstratiadis, 1984; Sittler et al., 1987; Zeitlin et al., 1987). In fact, excised intron lariats are protected from degradation in yeast mutants that lack debranching activity (Chapman and Boeke, 1991), indicating that the usual rapid degradation of excised introns occurs after debranching. Given these results, it is likely that the excised *Dl* introns accumulate predominantly as lariats. The relatively high levels of excised intron accumulation could reflect a specific property of the *Dl* introns or a more general property of the splicing machinery in *Drosophila* cells.

The excised *Dl* introns accumulate at highly localized foci within the nucleus. We have not detected any accumulation

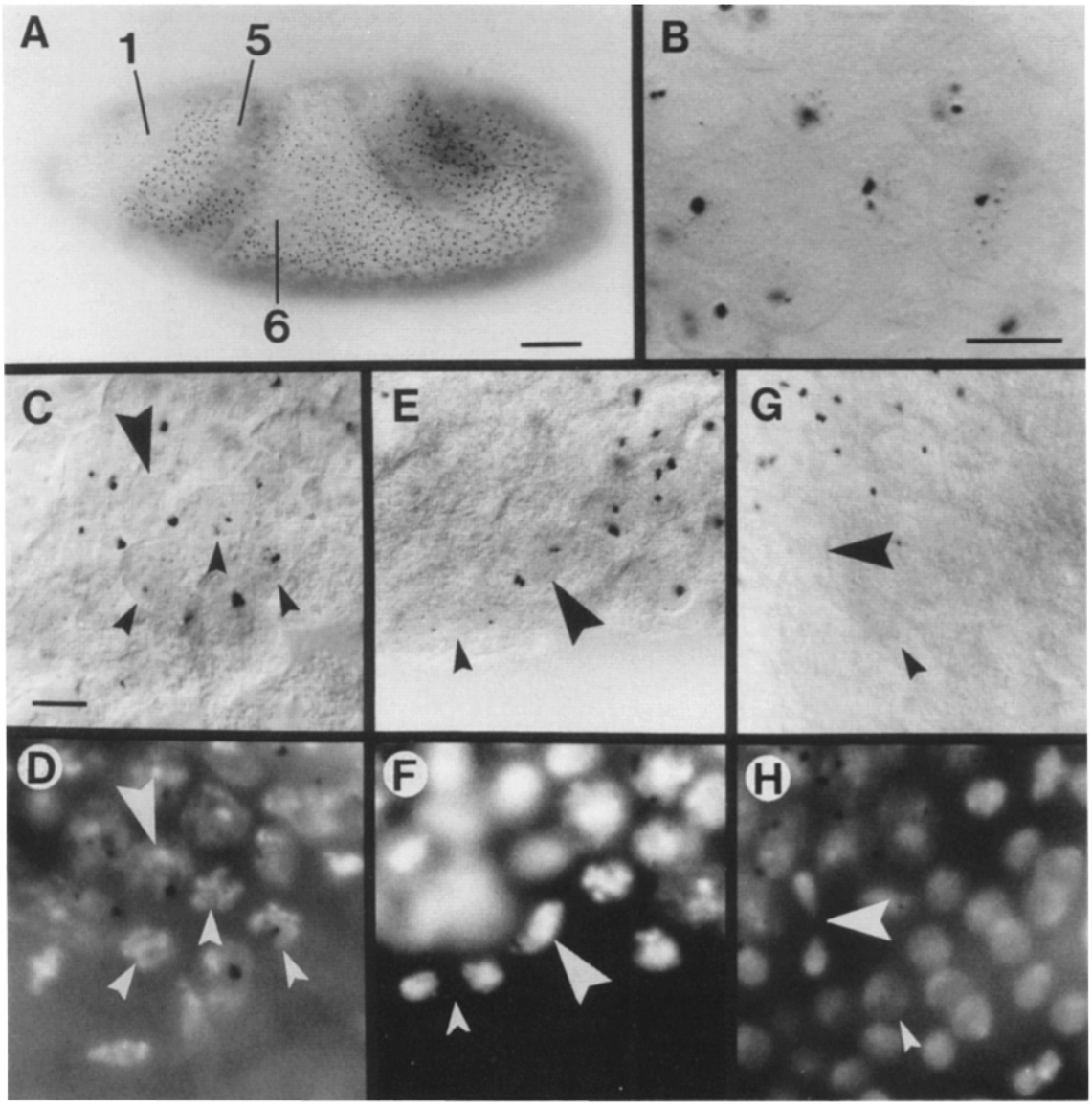

Figure 5. Excised intron localization is disrupted during mitosis. Embryos hybridized with the intron-specific probe were mounted in methyl salicylate (*A* and *B*) or stained with Hoechst 33342 and mounted in glycerol for fluorescence microscopy (*C–H*). (*A*) Embryo during early germ band extension. Hybridization is not detectable in regions corresponding to mitotic domains 1, 5, and 6 (Foe, 1989). (*B*) Nuclei within mitotic domain 5 in an embryo slightly younger than the embryo shown in *A*. Numerous small "dots" of hybridization are apparent in some nuclei. (*C* and *D*) Nuclei in early prophase (*large arrow*) or late prophase (*three small arrows*) of mitotic cycle 14. The numerous small "dots" are apparent only in late prophase nuclei. (*E* and *F*) Nuclei in metaphase (*large arrow*) or anaphase (*small arrow*) of mitotic cycle 14. Four foci of hybridization are present in each nucleus. At anaphase, two foci are present at each pole. (*G* and *H*) Nuclei in early telophase (*large arrow*) or late telophase (*small arrow*) of mitotic cycle 14. Intron probe hybridization is no longer detectable. Bars: (*A*) 50 μm; (*B* and *C*) 5 μm.

of the excised introns outside of these foci during interphase, even in nuclei that contain four copies of the *Dl* gene. However, by using silver precipitation to enhance the visibility of the histochemical reaction product, we were able to detect dispersal of excised *Dl* introns during mitosis. This observation underscores the sensitivity of the in situ hybridization technique used, and it suggests that the excised introns are in some way immobilized during interphase. Since excised β-globin introns have been shown to be tightly associated with isolated nuclear matrices (Zeitlin et al., 1987), it is possible that the excised *Dl* introns are bound to an equivalent structure in vivo. The fact that the excised introns become dispersed at late prophase, just prior to the dispersal of many nuclear matrix–associated antigens (reviewed in Verheijen et al., 1988), supports this possibility.

Our observation that the number of *Dl* genes present within the nucleus determines the number of excised intron foci observed strongly suggests that the excised introns accumulate near the sites of *Dl* transcription within the nucleus. Without exception, embryonic nuclei that contain a single *Dl* gene exhibit a single focus of excised intron accumulation, while embryonic nuclei containing two, three, or four *Dl* genes exhibit two, three, or four foci, respectively. In imaginal discs, however, where homologous chromo-

somes are believed to be paired, nuclei that contain two *Dl* genes exhibit only one focus of excised intron accumulation, while nuclei that contain four *Dl* genes exhibit only two excised intron foci. The conclusion that the excised introns accumulate near sites of *Dl* transcription is further supported by the observation that the foci colocalize with condensing chromosomes during mitosis.

The accumulation of excised introns near sites of *Dl* transcription is consistent with previous electron microscopic and biochemical analyses of in vivo splicing in *Drosophila*. Beyer and Osheim (1988) used EM to characterize spliceosome assembly and intron removal from nascent transcripts in embryonic nuclei. These authors found that introns located >4 kb upstream of a polyadenylation site are frequently removed cotranscriptionally. Similar results have been reported by LeMaire and Thummel (1990), who used biochemical techniques to analyze splicing at the *E74A* gene of *Drosophila*. It therefore seems likely that the accumulation of excised introns near sites of *Dl* transcription is a consequence of splicing being completed before the *Dl* pre-mRNA is released from the DNA template.

Recently, Shermoen and O'Farrell (1991) have reported the use of intron probes to follow transcription of the *Ultrabithorax (Ubx)* gene through the cell cycle in *Drosophila* embryos. These authors observed two foci of intron accumulation in interphase nuclei using a probe for the 5'-most *Ubx* intron and noted that these foci disappear during mitosis. Based on the assumption that an excised intron would be rapidly degraded, the authors propose that the disappearance of the foci reflects abortion of the nascent *Ubx* transcripts. Our results, along with the demonstrations of cotranscriptional splicing by Beyer and Osheim (1988) and LeMaire and Thummel (1990), suggest that the hybridization signal observed by Shermoen and O'Farrell (1991) could predominantly represent excised 5'-most *Ubx* introns, not nascent *Ubx* transcripts. If so, then much of the decrease in hybridization observed during mitosis might be the result of the dispersal of excised *Ubx* introns at prophase, as we have observed for the excised *Dl* introns.

Interphase Pairing of Homologous Chromosomes in Imaginal Nuclei

The ability to visualize the location of *Dl* genes within the nucleus allowed us to address whether homologous chromosomes are paired during interphase in diploid *Drosophila* cells. Our results reveal that *Dl* genes located on homologous chromosomes are colocalized in imaginal disc nuclei. This provides physical evidence for homologous chromosome pairing during interphase in imaginal cells, which previously has been inferred from genetic analyses (Lewis, 1954; Garcia-Bellido and Wandosell, 1978; Jack and Judd, 1979; Gelbart, 1982). The mechanism responsible for the pairing is still unknown, but its effect on gene regulation in *Drosophila* has been well-documented (Lewis, 1954; Kaufman et al., 1973; Jack and Judd, 1979; Babu and Bhat, 1981; Gelbart, 1982; Gelbart and Wu, 1982; Gubb et al., 1986). Interestingly, pairing-dependent genetic complementation, or "transvection" (Lewis, 1954), has not been observed in *Drosophila* embryos. This is consistent with our discovery that homologous *Dl* genes are physically separate in embryonic nuclei, suggesting that homologous chromosomes are not paired during early development.

Models for the Relationship between Nuclear RNA Processing and Transport

The "gene gating" (Blobel, 1985) and "translational translocation" (Urlaub et al., 1989) models for gene expression and RNA processing propose that the splicing machinery is associated with nuclear pores. The distribution of *Dl* intron accumulation sites within the nucleus demonstrates that *Dl* transcription and pre-mRNA processing do not require association with the nuclear lamina, and thus does not support these models. However, Spector (1990) has recently proposed a model for the organization of the nucleus that is consistent with our results. The model, based upon immunohistochemistry and ^{3}H-uridine labeling of nuclei, proposes that the splicing machinery is organized as a reticular network that extends throughout the nucleus (excluding nucleoli) and that actively transcribed genes are peripherally associated with this network. According to this model, the different sites of *Dl* intron accumulation within the nucleus represent different positions of the *Dl* gene along this reticular network.

The localization of excised *Dl* introns near sites of *Dl* transcription differs from the localization of cardiac α-actin and AChR introns in chick myoblast nuclei. Berman et al. (1990) have shown that the fifth intron of the α-actin pre-mRNA is widely distributed in myoblast nuclei, whereas the first intron of the AChR pre-mRNA is specifically localized around the nuclear periphery. (We note that the authors did not determine whether these distributions represented excised introns or unspliced pre-mRNA). We suggest that the different intranuclear distributions of *Dl*, α-actin, and AChR intronic sequences could reflect different rates of pre-mRNA splicing relative to nuclear transport. Since all of the *Dl* introns are more than 4 kb upstream of the predominant polyadenylation site (Kopczynski et al., 1988; Haenlin et al., 1990), the accumulation of excised *Dl* introns near sites of *Dl* transcription is probably a consequence of splicing being completed on the nascent pre-mRNA (discussed above). In contrast, the 3' end of the fifth α-actin intron is less than 800 bp from the polyadenylation site (Chang et al., 1985). If the rates of pre-mRNA splicing in chick myoblasts and *Drosophila* embryos are similar, then the fifth α-actin intron would not be removed until after the pre-mRNA is released from the DNA template. The final splicing of the α-actin pre-mRNA might then occur as the immature transcript is transported to the nuclear envelope, resulting in a wider distribution of α-actin intronic sequences. The perinuclear distribution of the first AChR intron suggests that this intron is not removed from the pre-mRNA until after transport to the nuclear envelope is completed. This could be the result of inefficient recognition of this intron by the splicing machinery, perhaps due to unusual secondary structure or nonconsensus splice sites.

The mechanism by which pre-mRNAs and excised introns are selectively retained within the nucleus is currently unknown. It has been shown if the 5' and 3' splice sites flanking an intron are destroyed, the intron-containing pre-mRNA is exported to the cytoplasm (Chang and Sharp, 1989; Legrain and Rosbash, 1989). Similarly, pre-mRNAs are exported from the nucleus if spliceosome assembly in yeast is disrupted (Legrain and Rosbash, 1989; Ruby and Abelson, 1991). These results have led to a "spliceosome retention" model for the selectivity of RNA export (Green and Zap, 1989), which proposes that pre-mRNAs are retained within

the nucleus through their association with spliceosomes. Based upon the apparent immobilization of excised *Dl* introns within the nucleus, we suggest that introns could serve to anchor pre-mRNAs within the nucleus until splicing is completed. The ability of introns to serve as "anchors" could be based on their association with spliceosomes, which may in turn be associated with the nuclear matrix (Verheijen et al., 1988; Carmo-Fonseca et al., 1991). According to this "intron anchor" model, a pre-mRNA that is not spliced cotranscriptionally would be free to be transported toward the nuclear envelope until an intron became assembled with spliceosomal snRNPs. The pre-mRNA would then be immobilized until the intron was excised, after which transport would proceed until another intron became bound by snRNPs. This model is consistent with the reported accumulation of EBV pre-mRNA along nuclear "tracks" that extend from the site of transcription to the nuclear envelope (Lawrence et al., 1989).

The ability to visualize excised *Dl* introns within the nucleus provides a system to test models relating nuclear structure to RNA processing and export. For example, the "intron anchor" model predicts that introns that are inefficiently recognized by the splicing machinery should be localized at points further along an export pathway than introns that are rapidly excised from a pre-mRNA. This prediction could be tested by introducing mutations near the 5′ or 3′ splice junctions of a *Dl* intron and subsequently assaying the nuclear distribution of this intron relative to the other *Dl* introns. Such experiments could provide important insight into the relationships between RNA transcription, RNA splicing, and RNA export as they occur within the highly structured nucleus.

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