

Intron-dependent Recruitment of Pre-mRNA Splicing Factors to Sites of Transcription

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Abstract. We have examined the nuclear localization of transiently and stably expressed nascent RNA transcripts containing or lacking introns in order to determine if the spatial association of RNA transcripts and pre-mRNA splicing factors in nuclei is random or functionally significant. Our findings show that the association between nascent RNA and splicing factors in the nucleus is intron-dependent when the RNAs are either transiently or stably expressed. Furthermore, our data indicate that splicing factors are recruited to the tran-

scription sites. The presence of both pre- and mRNA at these locations suggest that pre-mRNA splicing occurs at the sites of transcription. In addition, electron microscopic examination of the highly active transcription sites has revealed a granular appearance which closely resembles, but is functionally different from, interchromatin granule clusters. Our findings demonstrate that the nucleus is highly organized and dynamic with regard to the functions of transcription and pre-mRNA splicing.

PRE-mRNA splicing, which involves intron excision and exon ligation, is an essential posttranscriptional modification for the majority of RNA transcripts transcribed by RNA polymerase II. Extensive biochemical and molecular approaches have shown that pre-mRNA splicing is a complex multi-step process (for reviews see Green, 1991; Sharp, 1994) which may involve up to 50–100 proteins, some of which are snRNP components (Reed, 1990). The development of an efficient *in vitro* splicing system (Krainer et al., 1984) significantly advanced our understanding of the basic steps involved in conventional pre-mRNA splicing. The specific roles of many of the components participating in the splicing reaction, including snRNPs and non-snRNP splicing factors, have been extensively investigated (for review see Green, 1991). In spite of an increased understanding of the biochemical mechanisms involved in pre-mRNA splicing, a clear picture of the spatial and temporal organization of pre-mRNA splicing in the cell nucleus has not yet been achieved.

Earlier studies have shown that many of the well characterized splicing factors including the major snRNPs and certain non-snRNP splicing factors such as SC35, SF2/ASF, and other SR proteins are localized in a speckled nuclear pattern in addition to being diffusely distributed in the nucleus (for a review see Spector, 1993). When the localization of splicing factors was examined at the electron microscopic level, the speckled distribution pattern ob-

served at the light microscopic level was found to correspond to two distinct nuclear structures, interchromatin granule clusters, and perichromatin fibrils (Spector et al., 1991). Both of these components have been previously defined according to their morphological characteristics (for a review see Fakan and Puvion, 1980). Interchromatin granule clusters do not incorporate [³H]uridine after short pulses of labeling (for a review see Fakan and Puvion, 1980), and do not immunolabel with anti-DNA antibodies (Turner and Franchi, 1987). These findings suggest that interchromatin granule clusters are unlikely to be the sites of active transcription. Instead, they have been proposed to be sites of storage and/or reassembly of snRNPs and non-snRNP splicing factors (Jiménez-García and Spector, 1993; O'Keefe et al., 1994; Spector et al., 1993). In contrast, perichromatin fibrils incorporate [³H]uridine after short pulses (for a review see Fakan, 1994) suggesting that they represent nascent transcripts, and therefore are the sites of active transcription.

The elaborate organization of splicing factors in a speckled pattern has been shown to reflect the transcriptional and splicing activities of the cell (Jiménez-García and Spector, 1993; Spector et al., 1993; O'Keefe et al., 1994). When RNA polymerase II activity is inhibited upon treatment with α -amanitin, the distribution of splicing factors is reorganized into larger and fewer round structures. When examined using electron microscopy, splicing factors are primarily concentrated in interchromatin granule clusters, whereas, perichromatin fibrils are significantly decreased in these cells (Spector et al., 1993). In addition, microinjection into cells of oligonucleotides or antibodies which inhibit pre-mRNA splicing *in vitro* results in a similar redis-

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tribution of splicing factors (O'Keefe et al., 1994). The reorganization of splicing factors due to the lack of either pre-mRNA splicing substrate or functional splicing components (snRNPs)¹ suggests that the localization of splicing factors at perichromatin fibrils is a result of the requirement of splicing factors at the sites of transcription. Furthermore, when cells are infected with adenovirus 2, splicing factors are recruited to the introduced highly active transcription sites as detected by immunostaining with multiple antibodies which specifically recognize splicing or transcription factors (Jiménez-García and Spector, 1993; Pombo et al., 1994). These findings suggest that splicing factors shuttle between storage and/or reassembly sites (interchromatin granule clusters) and sites of active transcription and pre-mRNA splicing (Jiménez-García and Spector, 1993; Pombo et al., 1994). However, other studies have not found splicing factors to be localized within these viral structures during the infection (Zhang et al., 1994), or at the late phase of the infection (Bridge et al., 1993, 1995).

To analyze the spatial and temporal organization of pre-mRNA splicing in mammalian cells, several individual RNA species transcribed from endogenous templates have been localized and compared to the localization of splicing factors. The induced expression of *c-fos* transcripts was found to be closely associated with splicing factors both spatially and temporally suggesting a close link between transcription and splicing (Huang and Spector, 1991). Such a close association or colocalization between nascent RNA and splicing factors has also been observed for other endogenously expressed RNA transcripts such as fibronectin, neurotensin, collagen I α 1, and the Epstein Barr virus Bam W RNA (Xing and Lawrence, 1991; Xing et al., 1993, 1995). However, conflicting observations have been reported with regard to the colocalization of β -actin RNA and splicing factors (Zhang et al., 1994; Xing et al., 1995). In addition, the localization of an intron-less RNA and its relationship to the localization of splicing factors has thus far not been reported. Therefore, it is presently unclear whether the spatial association of splicing factors and nascent RNA transcripts is functionally specific or a random coincidence.

In this report, we have used transient and stable expression systems to determine (a) whether the spatial association of nascent RNA and splicing factors is functionally specific or merely a random coincidence; (b) whether splicing factors are recruited to the sites of transcription as suggested from studies using adenoviral infection; and (c) the ultrastructural features of the active transcription sites. We have found that the association of nascent RNA transcripts with splicing factors is intron-dependent during transient or stable expression. Splicing factors are recruited to the sites of active transcription of introduced intron-containing templates. In addition, electron microscopic analysis of highly active transcription sites has revealed a granular structure which closely resembles, but functionally differs from a typical interchromatin granule cluster. Together, these findings demonstrate a high degree of organization and signaling that takes place within the nucleoplasm. In addition, this study elaborates upon

1. *Abbreviations used in this paper:* CLSM, confocal laser scanning microscopy; DRB, 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole; snRNP, small nuclear ribonucleoprotein particle.

the functional significance of interchromatin granule clusters which were first described by Swift (1959).

Materials and Methods

Cell Culture and Fixation

HeLa cells were grown to subconfluence on glass coverslips in 35-mm-diam Petri dishes in DMEM supplemented with 10% FBS (GIBCO BRL, Gaithersburg, MD). Cells were maintained at 37°C with 10% CO₂. HeLa cells that express the stably integrated constructs encoding β -globin genomic or β -globin cDNA were grown in the same conditions with the addition of 200 μ g/ml G418. The inhibition of RNA polymerase II transcription was achieved by the addition of α -amanitin (50 μ g/ml for 5 h) or 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB) (25 μ g/ml for 3 h) to the culture medium.

Plasmids

Most constructs that we used were generous gifts from colleagues. Among them, constructs that expressed intron-less RNAs, β -galactosidase, and Adenoviral VA RNA, and an RNA with a partial intron, CMVTAT, were provided by Drs. Shobha Gunnery and Michael Mathews (Cold Spring Harbor Laboratory); intron-containing RNAs: β -globin genomic DNA (Caceres et al., 1994) was provided by Drs. Javier Caceres and Adrian Krainer, CGTAT (Ratnasabapathy et al., 1990) was provided by Drs. Shobha Gunnery and Michael Mathews, and β -tropomyosin minigene (Helfman et al., 1988) was provided by Drs. Wei Guo and David Helfman (Cold Spring Harbor Laboratory). β -Globin cDNA was inserted into the same parental plasmid as the β -globin genomic DNA using PCR fragments so that both constructs could be expressed under identical conditions.

Transfection

Expression constructs were transiently transfected into HeLa cells by electroporation (Sambrook et al., 1989). Briefly, subconfluent cells in a 100-mm culture dish were collected by trypsinization and mixed with 20 μ g of DNA including 7 μ g target DNA and 13 μ g sheared salmon sperm DNA. A 280- μ l mixture of cells in DMEM with 10% FBS and DNA was electroporated in an electroporator at 270 V and 960 μ FD. Cells were subsequently seeded onto glass coverslips in 35-mm-diam Petri dishes and were grown for either 7 or 24 h before being fixed for RNA or protein labeling. To establish a stable integration of a construct, cells were grown in selection medium containing 400 μ g/ml of G418 beginning at 36 h after the transfection.

In Vivo Incorporation of BrUTP

The in vivo transcription assay was performed according to published studies (Jackson et al., 1993; Wansink et al., 1993). Briefly, at 7 or 24 h posttransfection, cells were rinsed once with PBS and once with a glycerol-containing buffer (20 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 25% glycerol, 0.5 mM PMSF, and 0.5 mM EGTA). Cells were then permeabilized in the glycerol buffer containing 0.1% Triton X-100 at room temperature for 3 min. Subsequently, cells were incubated in transcription cocktail (100 mM KCl, 50 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 0.5 mM EGTA, 25% glycerol, 1 mM PMSF, 2 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 0.2 mM BrUTP and 25 U/ml RNasein) for 4.5 min at 37°C. At the end of the transcription reaction, cells were gently rinsed twice with PBS, and then fixed in 4% formaldehyde in PBS.

In Situ Hybridization

In situ hybridization was performed as described previously (Huang and Spector, 1991). Fixed cells were washed, 3 \times 15 min each in PBS and permeabilized in 0.5% Triton X-100 for 5 min at 4°C. Cells were then rinsed in PBS, 2 \times 15 min each and once with 2 \times SSC. For nuclease digestions, cells were incubated with either 100 μ g/ml of RNase A or 5 U/30 μ l of RNase-free DNase I (Boehringer Mannheim, Indianapolis, IN) at 37°C for 2 h and washed extensively in PBS before hybridization.

Probes were made by nick translation of specific DNA templates in the presence of biotin- or digoxigenin-labeled dUTP. 100–500 ng of labeled probe was dried in a Speed-vac, together with 20 μ g of *Escherichia coli*

tRNA and 5 μ g of sheared salmon sperm DNA. The pellet was resuspended in 10 μ l deionized formamide, heat-denatured at 75°C for 10 min and rapidly cooled in an ice water slurry. The final 20- μ l hybridization mixture (2 \times SSC, 10 mM Tris-HCl, pH 7.2, 1 mM EDTA, 5% dextran sulfate, 50% formamide [vol/vol], denatured probe, tRNA, and sheared salmon sperm DNA) was applied to each coverslip which was inverted onto an RNase-free glass slide and was sealed with rubber cement. Hybridization was carried out at 37°C in a humidified chamber overnight. After hybridization, cells were washed three times in 2 \times SSC and once in 1 \times SSC.

Signal Detection by Fluorescence Microscopy

Hybridizations with biotinylated probes were detected by incubating in FITC-conjugated avidin (Vector Labs., Burlingame, CA) at 2 μ g/ml in 4 \times SSC as described by Huang and Spector (1991). Cells were then extensively washed in 4 \times SSC at room temperature. Hybridizations with the digoxigenin-labeled probe were detected with FITC-conjugated anti-digoxigenin Fab' (Boehringer Mannheim) at a dilution of 1:20–1:40 in PBS. Cells were incubated in antibody for 1 h at room temperature and washed with PBS or PBS plus 0.1% Triton X-100.

Immunolabeling

After detection of the hybridization signal, cells were washed, 3 \times 10 min each, in PBS and incubated with anti-SC35 primary antibody (Fu and Maniatis, 1990) at a dilution of 1:1,000, anti-B'' (Habets et al., 1989) at a dilution of 1:5, anti-Sm antibody (Lerner et al., 1981) at a dilution of 1:1,000, or anti-BrdU antibody (Boehringer Mannheim) at a dilution of 1:5 for 1 h at room temperature. Cells were rinsed in PBS, and then incubated with Texas red-conjugated goat anti-mouse antibody at a dilution of 1:30 for 1 h at room temperature, followed by three washes in PBS. The coverslips were mounted onto glass slides with mounting medium containing 90% glycerol in 0.2 M Tris-base (pH 8.0) with 1 μ g/ml paraphenylenediamine as an antifading agent. Cells were examined with a Nikon FXA microscope equipped with epifluorescence and differential interference contrast optics. In double-labeling experiments, red and green fluorescence was simultaneously detected in 0.5 μ m optical sections using a Leica TCS 4D confocal laser scanning microscope. Images were recorded with a Focus Graphics image recorder.

Morphological Characterization by Electron Microscopy

To examine the nuclear regions involved in the transcription and splicing of the transiently expressed RNAs at the EM level, we correlated the localization of the RNA at the light microscopic level to the immunodetection of the same region at the EM level using anti-SC35 antibody. 8 h posttransfection, cells were fixed in 4% paraformaldehyde with 0.05% glutaraldehyde in PBS and were in situ hybridized to the corresponding probes as described above. The fluorescent hybridization signals of cells localized on gridded coverslips were photographed. Subsequently, the coverslip was floated off of the slide and the cells were fixed in 0.5% glutaraldehyde for 20 min and washed in PBS containing 0.3 M glycine. Cells were then dehydrated by incubation in a series of ascending concentrations of ethanol and embedded in LR White resin at 60°C for 48 h. Thin sections of the same cell, previously photographed by fluorescence microscopy, were immunogold labeled with antibodies specifically recognizing SC35 and post-stained by the EDTA regressive method (Bernhard, 1969) which revealed RNP-enriched structures. Sections were examined using a Hitachi H-7000 transmission electron microscope operated at 75 kV.

Results

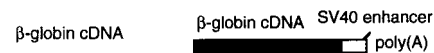
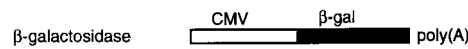
To examine whether the spatial association between nascent RNA transcripts and splicing factors in the nucleus is functionally specific, we have examined the localization of several transiently and stably expressed intron-containing RNAs or intron-less RNAs and compared their localization to the distribution of splicing factors in the same cells. If the colocalization between RNA and splicing factors was merely a random coincidence, intron-containing and intron-less RNAs would have an equal chance to associate with splicing factors. However, if the colocalization of na-

scient RNA and splicing factors represents a functional interaction, one would expect such a colocalization to be intron-dependent. Three classes of templates were used in this study. They were (1) intron-less RNAs (β -galactosidase, β -globin cDNA, and Adenoviral VA RNA, an intron-less RNA transcribed by RNA polymerase III [Gunnery et al., 1992]); (2) RNA with a partial intron (CMVTAT), and (3) intron-containing RNAs (β -globin genomic DNA [Caceres et al., 1994], CGTAT [Ratnasabapathy et al., 1990], and β -tropomyosin minigene [Helfman et al., 1988]) (Fig. 1). The intron-containing β -globin genomic and intron-less β -globin cDNA were expressed from the identical parental plasmid.

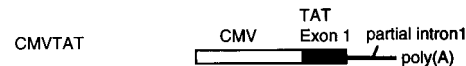
RNA Transcripts without Introns Do Not Associate with Splicing Factors

HeLa cells were transiently transfected with constructs expressing intron-less RNAs (β -galactosidase or β -globin cDNA). 7 h after transfection, cells were subjected to in situ hybridization using the corresponding probes which were derived from the transfected construct. The same cells were also immunolabeled with a monoclonal antibody specifically recognizing SC35. The localization of the RNA and SC35 was observed in 0.5 μ m optical sections obtained using a confocal laser scanning microscope (CLSM). In situ hybridization demonstrated that both β -galactosidase (Fig. 2 a) and β -globin (Fig. 2 d) mRNA were localized to many discrete regions in the nucleus that were visualized as green dots. The number of dots per nucleus varied from four or five up to one hundred. Cytoplasmic RNA hybrid-

(-) Intron



Partial Intron



(+) Intron

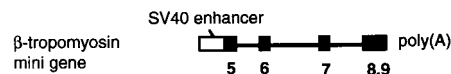


Figure 1. Diagram illustrating the constructs used in transient transfections. The β -galactosidase and β -globin cDNA constructs do not contain intron sequences. The CMVTAT construct contains a partial intron. The adenoviral VA construct contains neither an intron sequence nor a poly(A) tail and is transcribed by RNA polymerase III. The CGTAT gene, β -globin, and β -tropomyosin minigene gene constructs contain intron sequences. Both intron-containing β -globin genomic and intron-less β -globin cDNA are inserted into the same expression vector.

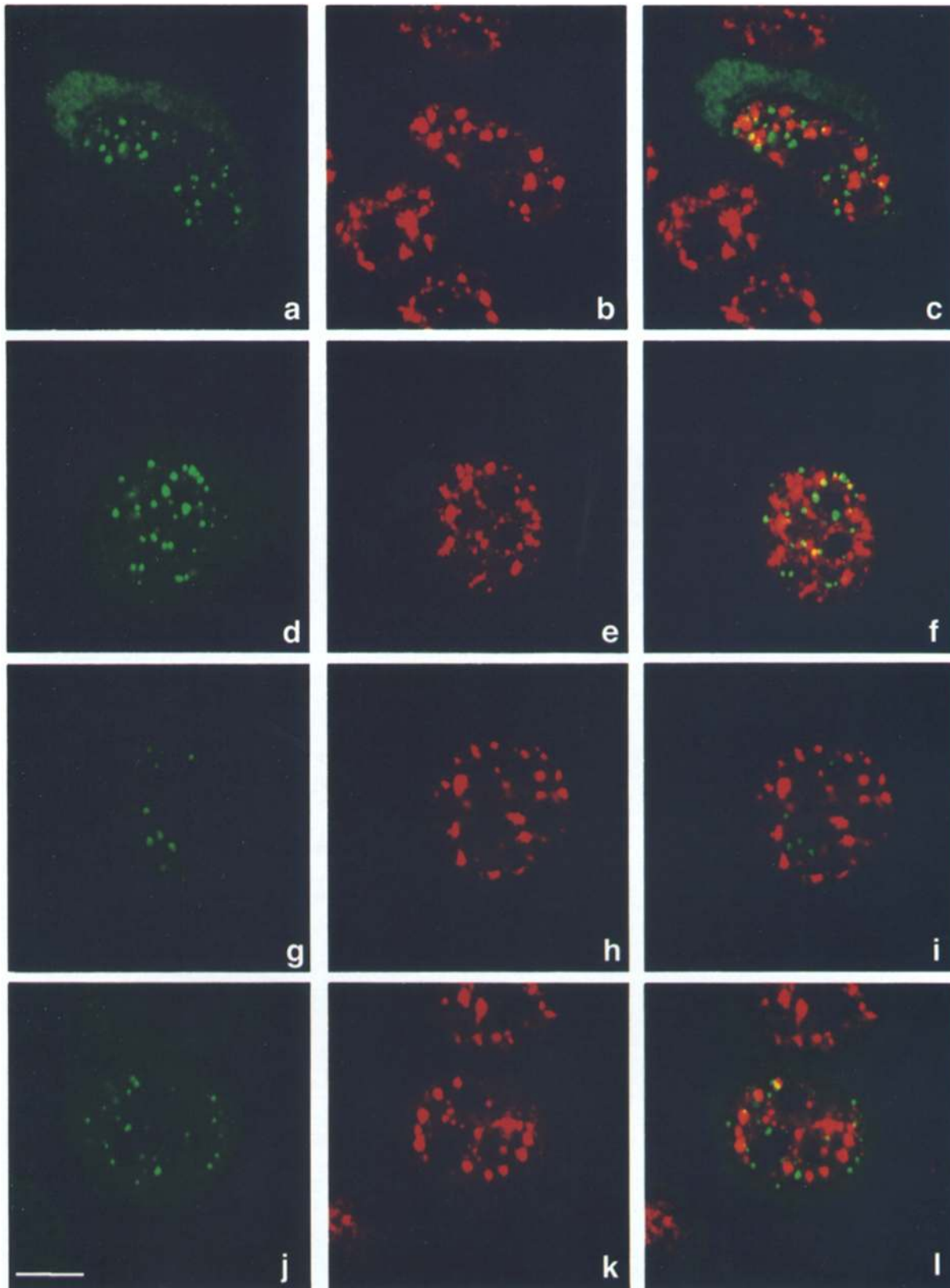


Figure 2. The majority of transiently expressed intron-less RNAs or RNAs with a partial intron are not spatially associated with splicing factors in the cell nucleus. HeLa cells were transiently transfected with expression vectors which encoded for RNA transcripts without introns: β -galactosidase (*a-c*), β -globin cDNA (*d-f*), or RNA transcripts containing a partial intron, CMVTAT (*g-i*), or adenoviral VA RNA transcribed by RNA polymerase III (*j-l*). The RNAs were detected by fluorescence in situ hybridization (FISH) with biotinylated probes 8 h after transfection (*a, d, g, and j*). The splicing factor SC35 was localized in the same cells using a monoclonal antibody specifically recognizing SC35 (*b, e, h, and k*). Simultaneous detection of both RNAs and SC35 (*c, f, i, and l*) showed little to no colocalization between the RNAs and splicing factors. The bar represents 10 μ m.

ization was also observed demonstrating that the RNA was being transported (Fig. 2 *a*). The amount of RNAs detected in the cytoplasm varied among cells expressing the same constructs and cells expressing different constructs. This variability may be due to the different expression levels of the same construct and/or differences in the stability of RNAs expressed from different constructs. Sometimes, visualization of cytoplasmic RNA also depended upon the focal plane of the cell which was examined. Optical sections derived from the top of the cells usually revealed very little of the cytoplasm whereas optical sections derived from the bottom of the cells displayed a much larger cytoplasmic area. The distribution of the splicing factor SC35 demonstrated a typical speckled localization in the cell nucleus (Fig. 2, *b* and *e*). Simultaneous examination of the localization of both the RNA and SC35 using CLSM revealed little spatial association between the β -galactosidase or β -globin mRNA signals and the localization of SC35 in a majority of the cases, since both green (RNA) and red (SC35) signals were mostly observed in different regions (Fig. 2, *c* and *f*). We also examined the expression of an HIV Tat RNA lacking a 3' splice site (Fig. 2 *g*). We were interested in knowing whether a 5' splice site alone would elicit an association between the Tat RNA and splicing factors. The transcript was comprised of exon 1 of HIV Tat and a partial intron of 300 bp including the 5' splice site. When examined by CLSM, we did not observe a spatial association between the localization of this RNA and the splicing factor SC35 (Fig. 2 *i*). Furthermore, we have also examined the expression of adenoviral VA RNA, an RNA polymerase III transcript. VA RNA does not contain any introns or a poly(A) tail. The localization of this RNA was observed in round dots in the nucleus (Fig. 2 *j*). Similar to that observed with the previous three RNAs, detection of VA RNA (Fig. 2 *j*) and splicing factors (Fig. 2 *k*) in the same cell revealed little association between the RNA and splicing factors (Fig. 2 *l*).

RNA Transcripts Containing Introns Are Closely Associated with Splicing Factors

We were next interested in examining the localization of RNAs that contain introns and their relationship with splicing factors in the nucleus. Constructs expressing RNA transcripts for β -globin, CGTAT, and a β -tropomyosin minigene (Fig. 1) were transfected into HeLa cells. The expression of the RNA and the localization of the splicing factors in the same cells were simultaneously detected by CLSM (Fig. 3). The localization of the majority of the RNA signal (~85%) from all three constructs were colocalized with the splicing factor SC35 (Fig. 3, *c*, *f*, and *i*). Such a colocalization was also observed when other splicing factors such as U2 snRNPs were examined using a monoclonal antibody specifically recognizing the U2 B'' protein (Habets et al., 1989) (Fig. 3 *l*) or other anti-snRNP antibodies (Sm, m₃G) (data not shown). The size and shape of the RNA localization signals varied in different cells or even in the same cell. Some of the intron-containing RNAs were localized as large clusters in the nucleus (Fig. 3, *g* and *j*). The corresponding nuclear regions were also occupied by similar size and shape clusters of splicing factors which were larger than a native SC35 speckle (Fig.

3, *h* and *k*, *arrows*). In addition, the fluorescence signal of the SC35 immunostaining in the speckles containing the transiently expressed RNAs (Fig. 3, *h* and *k*, *arrows*) appeared brighter in intensity suggesting a reorganization or recruitment of splicing factors to the location of the nascent RNA transcripts. However, we did not detect a core of splicing factors within a larger region of RNA as reported for the localization of poly(A)⁺ RNA (Carter et al., 1993).

Control experiments in which cells were transfected with carrier DNA alone, and then hybridized with probes derived from either intron-less or intron-containing RNA expression constructs resulted in no hybridization signals compared to 5–40% of the cells showing a positive hybridization signal when transfected with each of the seven constructs above. Furthermore, when cells were pretreated with RNase A for 2 h at a concentration of 200 μ g/ml before the hybridization, no signal was observed.

We have examined the localization of RNA expressed from seven different constructs and we have found that the association between the localization of nascent RNA transcripts and splicing factors is dependent upon the presence of a complete intron. To ensure that these observations were representative, we have examined a large number of cells that expressed these constructs and evaluated the association of their localization with splicing factors by CLSM. Over 400 sites of RNA localization were examined for the expression of each construct. The quantitative analysis is summarized in Fig. 4. The percentage of RNA localization sites that were colocalized, not colocalized, or partially colocalized with SC35 were plotted on the Y axis and the RNAs are indicated on the X axis. This histogram clearly demonstrated that the localization of intron-less RNA or RNA with a partial intron did not overlap with the localization of SC35 in over 80% of the cases examined. In contrast, over 85% of the localization sites of intron-containing RNA were found to be colocalized with SC35. These findings clearly show that the association between the localization of nascent RNA transcripts and splicing factors is intron-dependent and not random. Therefore, the colocalization is likely to be the result of a functional interaction between pre-mRNA and splicing factors.

Stably Expressed Intron-containing RNA Also Colocalizes with Splicing Factors

To confirm the observations made in the transient transfection assays, we have also evaluated the localization of stably expressed intron-less vs intron-containing RNAs and their association with splicing factors. The expression of RNA from a stably integrated β -globin cDNA or β -globin genomic DNA was examined. The localization of β -globin mRNA made from a stably integrated β -globin cDNA was not associated with SC35 (Fig. 5 *c*, *arrow*) in over 80% of the cells examined. In contrast, we found that β -globin RNA made from the genomic DNA was colocalized with the splicing factor SC35 (Fig. 5 *f*, *arrow*) in 98% of the cells examined. Similar to our observations of transiently expressed RNAs, the shape of the region containing the stably expressed RNA resembled the localization of the splicing factors at that site in a majority of the observed cells

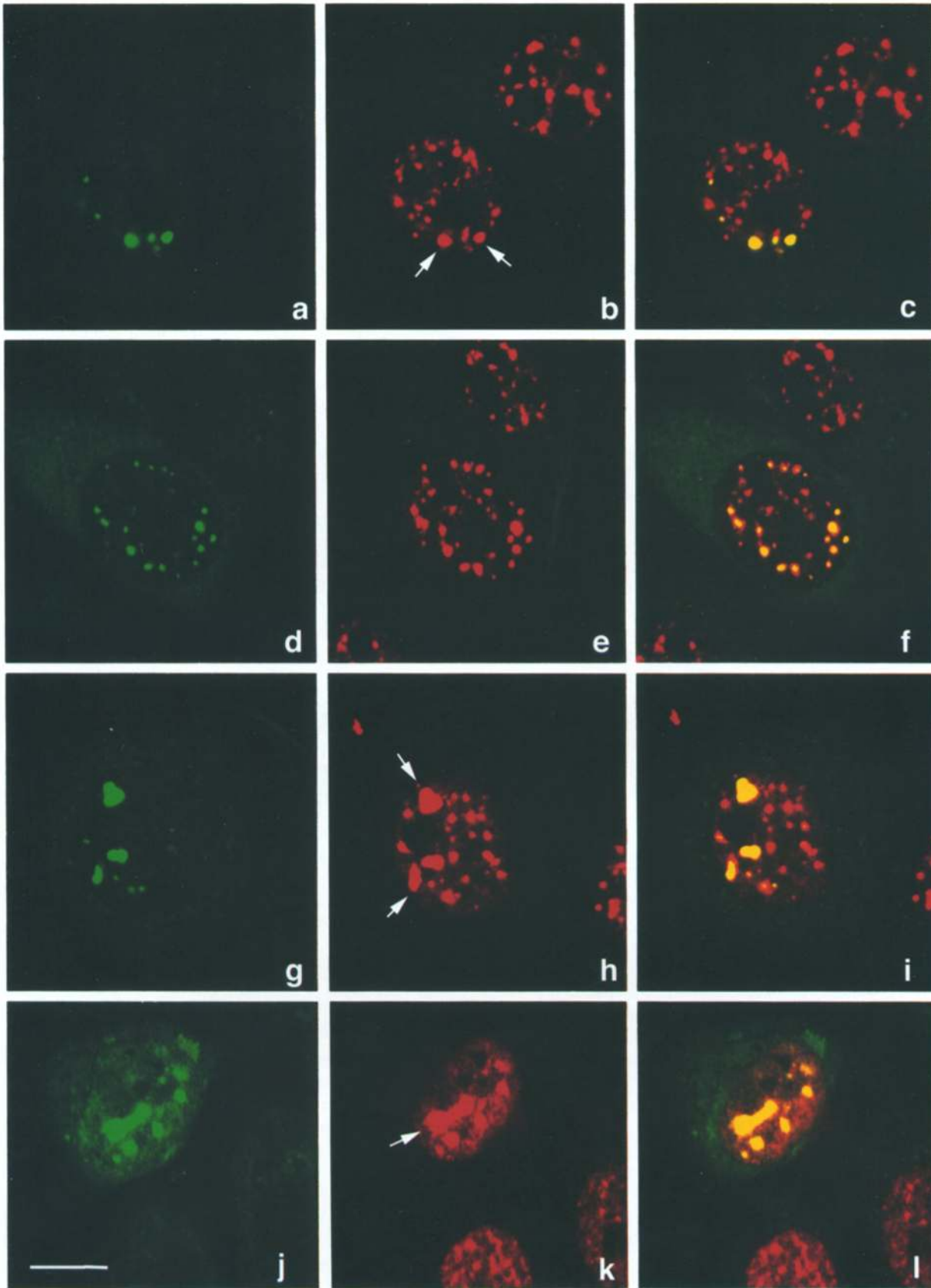


Figure 3. The transiently expressed RNA transcripts containing introns are spatially associated with splicing factors. HeLa cells were transfected with expression vectors which encode for intron-containing RNAs including β -globin (*a-c*), CGTAT (*d-f*), or β -tropomyosin minigene (*g-l*). The localization of these RNAs was examined by FISH with biotinylated probes 8 h after transfection (*a*, *d*, *g*, and *j*). The localization of splicing factors in the same cells was detected by immunostaining with a monoclonal antibody specifically recognizing SC35 (*b*, *e*, and *h*), or U2 snRNP B'' protein (*k*). RNA and splicing factors colocalized to the same nuclear regions (*c*, *f*, *i*, and *l*). Note that in many cases both signals were detected as large clusters (*arrow*). The bar represents 10 μ m.

The association of transcripts and splicing factors

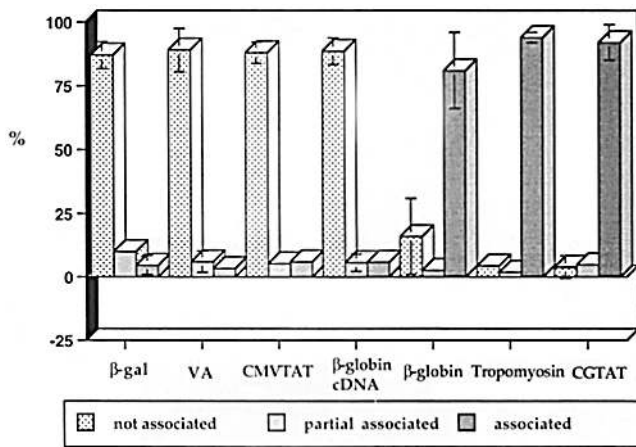


Figure 4. Quantitative analysis demonstrating that the spatial association between the nascent RNA transcripts and splicing factors in the nucleus is intron-dependent. The constructs are indicated on the X axis and the percentage of RNA localization sites that colocalized, did not colocalize, or partially colocalized with SC35 are plotted on the Y axis. Four hundred transcription sites were analyzed in each case.

(i.e., size and shape were the same) (compare the signal at the arrows in Fig. 5, *e* and *f*). The intron-dependent colocalization between stably expressed nascent RNA transcripts containing introns and splicing factors further supports the idea that such an association is functionally specific.

The Localization of the RNA Represents the Site of Transcription

We were next interested in determining whether the localization of transiently expressed RNA represented the sites of transcription. To address this question, we have examined the incorporation of BrUTP in transfected cells, after a short pulse, and tried to correlate the sites of transcription with the sites of RNA localization. If we could detect active incorporation of BrUTP at the same nuclear loci where the RNA was localized, this would indicate that these sites were the sites of transcription. 8 h after transfection, cells were incubated in a transcription mixture containing BrUTP for 4.5 min as described in Materials and Methods. Simultaneous detection of the localization of RNA (Fig. 6, *a* and *d*) and the incorporation of BrUTP (Fig. 6, *b* and *e*) revealed that the localization of the RNA produced by the transfected templates corresponded to the sites of active transcription. Such a colocalization was observed for both intron-less β -globin mRNA (Fig. 6 *c*) and intron-containing β -tropomyosin minigene RNA (Fig. 6 *f*). In some cells that expressed intron-containing RNA, the level of transcription of the exogenous templates was so high that an elevated incorporation of BrUTP was observed (Fig. 6 *e*, *arrows*) further supporting that the sites of the RNA localization were the sites of transcription. Variations in the BrUTP incorporation at different loci were also observed (Fig. 6, *b* and *e*) suggesting that there were differences in the extent of transcription among the differ-

ent templates in a single cell. Alternatively, some of the transcription sites may have contained multiple copies of the transfected templates. In addition, in many cases, we have observed the localization signal of the RNA to be larger than the BrUTP incorporation signal. One explanation of this observation is that the localization of the RNA may represent both RNAs that were made during the 4.5 min pulse of BrUTP as well as some of those that were made before the labeling, whereas, the signal for BrUTP incorporation only represented the RNA that was made during the 4.5-min of pulse labeling.

The Localization of Intron-containing RNA Represents the Site of Pre-mRNA Splicing

Since the sites of RNA localization represented the sites of transcription, we were interested in determining if pre-mRNA splicing occurred at the sites of transcription. To address this question, we investigated whether the RNA localization clusters contained both pre-mRNA and mRNA since the sites of pre-mRNA splicing should have both splice precursors (pre-mRNA) and spliced products (mRNA). The expression of the β -tropomyosin minigene was used as an example to dissect the components of the localized RNA. Introns 6 and 7 were subcloned and used as probes to specifically hybridize to β -tropomyosin pre-mRNA (Fig. 7). Splice junction probes were used to specifically hybridize to the mRNA (Fig. 7). The splice junction probe was a biotinylated 24-mer oligonucleotide, half of which was complementary to 12 nucleotides at the 3' end of exon 5, and the other half was complementary to 12 nucleotides at the 5' end of exon 6 (Fig. 7). Only when exons 5 and 6 are ligated, can this probe form a stable duplex with the target mRNA and therefore give a hybridization signal under the relatively high hybridization stringency used in this study.

Cells expressing the β -tropomyosin minigene RNA were simultaneously hybridized to intron-specific probes which were localized by Texas red-conjugated anti-digoxigenin (*red*) (Fig. 8 *b*) and to the splice junction probes which were localized by FITC-conjugated avidin (*green*) (Fig. 8 *a*). When cells were simultaneously scanned by CLSM, the pre-mRNA and the mRNA appeared to occupy the same nuclear regions (Fig. 8 *c*) demonstrating that pre-mRNA splicing took place in those regions. A splice junction probe spanning exons 5 and 8, which represented ~50% of the spliced product for this RNA, provided similar results as that observed with the splice junction exon 5 to 6 probe described above (data not shown). Hybridization with 12-mer oligonucleotides complementary to either the 3' end of exon 5 or the 5' end of exon 6 or exon 8 at the same stringency used for the splice junction probes did not result in specific signals, suggesting that the observed signals with the splice junction probes represented mRNA (data not shown). In some cells where the expression of the gene was extremely high, such that all of the nuclear regions enriched in splicing factors were found to be colocalized with the β -tropomyosin RNA, pre-mRNA was also detected in the cytoplasm with the intron-specific probe (Fig. 8, *d* and *f*). The presence of unspliced RNA in the cytoplasm suggested that the splicing machinery was saturated by the overexpression of the transfected

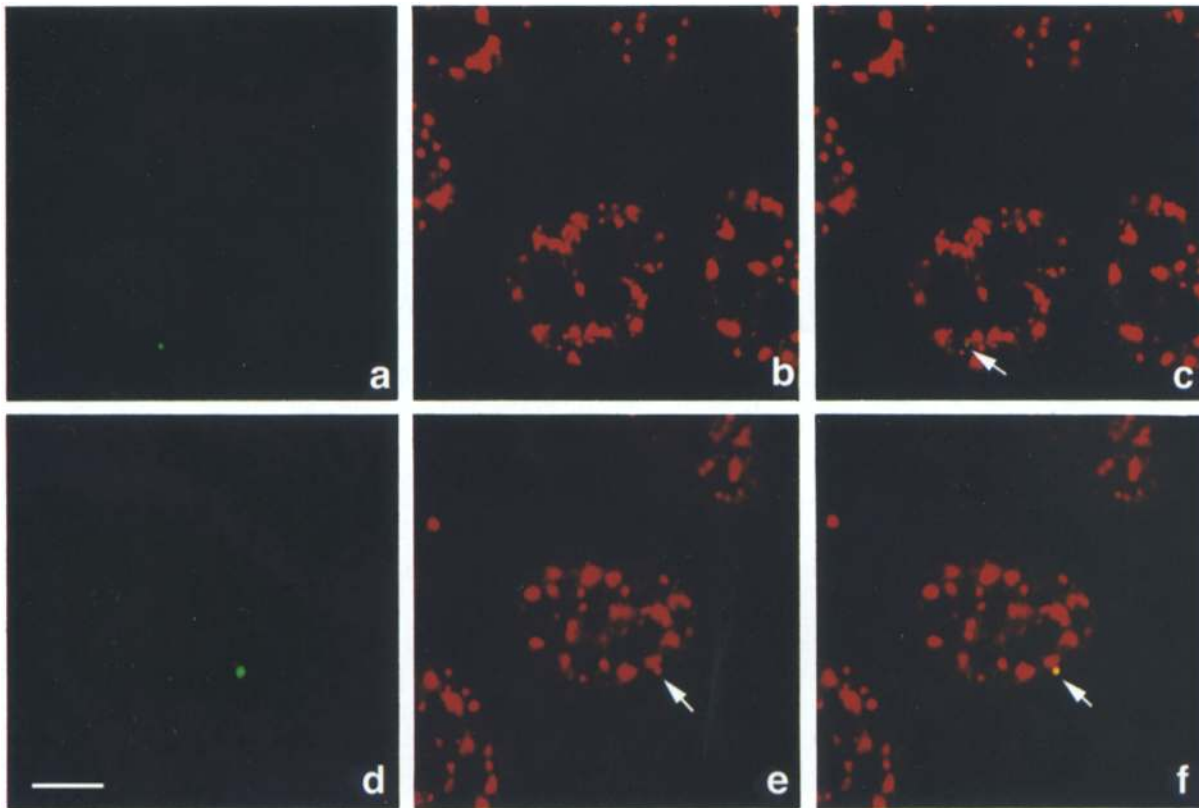


Figure 5. The intron-dependent association between nascent RNA transcripts and splicing factors is also observed in stable cell lines. Intronless β -globin cDNA (*a-c*) and intron-containing β -globin genomic DNA (*d-f*) were stably integrated into the HeLa cell genome. The RNAs were detected by fluorescence in situ hybridization with biotinylated probes (*a* and *d*). The localization of the splicing factor SC35 in the same cells was detected by immunostaining with monoclonal antibody specifically recognizing SC35 (*b* and *e*). The localization of β -globin mRNA transcribed from the β -globin cDNA construct was not spatially associated with SC35 in the majority of cells examined (*arrowhead* in *c*). In contrast, the localization of β -globin RNA transcribed from the β -globin genomic construct colocalized with SC35 (*arrowhead* in *f*). The bar represents 10 μ m.

templates and unable to splice all the RNAs that were made. Alternatively, a putative nuclear retention factor responsible for keeping unspliced pre-mRNA in the nucleus may have become saturated.

We have also examined the fate of the transiently expressed RNA in the nucleus by treating cells with the transcription inhibitor, α -amanitin 8 h after transfection. When cells were treated with 50 μ g/ml of α -amanitin for 5 h, the majority of the transiently expressed RNA, both intron-containing (Fig. 8 *g*) or intron-less RNA (data not shown), were only observed in the cytoplasm of the transfected cells. Little to no RNA was detected in the nucleus suggesting that these RNAs can be chased into the cytoplasm upon the inhibition of transcription. This finding also suggested that the large clusters of RNA localization observed for the expression of certain intron-containing RNAs was not due to an accumulation of RNA because of an alteration in RNA transport. Furthermore, this finding demonstrated that RNA transport was not blocked when RNA transcription was inhibited. The splicing factors in the transfected and nontransfected cells that were treated with transcription inhibitor displayed a characteristic phenotype of rounded large clusters (Fig. 8 *h*) typically observed after inhibition of RNA polymerase II (Spector et

al., 1993; Huang et al., 1994). However, it is not possible to distinguish whether a subset of splicing factors in transfected cells remain at the previously active transcription sites. Interestingly, when cells were treated with DRB, a transcription inhibitor (Sehgal et al., 1976) as well as a kinase inhibitor (Zandomeni and Weinmann, 1984), both intron-containing and intron-less RNAs were not chased out of the nucleus (data not shown). In this case, the RNAs were colocalized with splicing factors in large clusters. This observation suggested that DRB affects other processes in the cells in addition to the inhibition of RNA polymerase II activity.

Ultrastructural Analysis of the Association of Splicing Factors and the Expression of Intron-containing RNA

We next determined if the sites of transcription and pre-mRNA splicing of the RNAs generated from the transfected DNA templates corresponded to a specific nuclear substructure. Transfected cells were examined by fluorescence in situ hybridization and the localization of the RNA signal was photographed. The marked cells were then prepared for immunoelectron microscopy. The nuclear structure that corresponded to the hybridization signal was

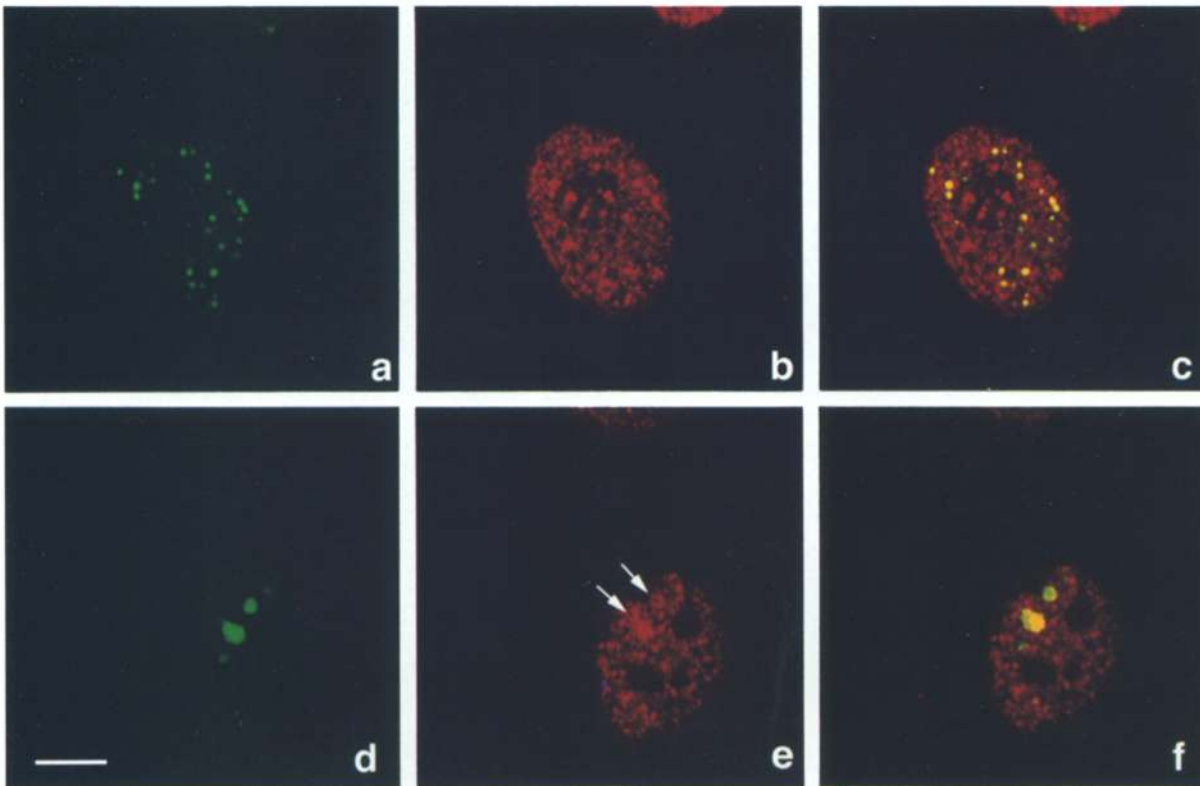


Figure 6. The localization of the transiently expressed RNAs represents the sites of transcription. The incorporation of BrUTP for 4.5 min in cells transfected with either a construct expressing an intron-less RNA (β -globin; *a-c*) or an intron-containing RNA (β -tropomyosin minigene; *d-f*) was examined by immunostaining with a monoclonal antibody specifically recognizing BrUTP (*b* and *e*). The localization of the RNA was examined by FISH (*a* and *d*). The bar represents 10 μ m.

identified based upon its morphological appearance, position in the nucleus, as well as its immunogold labeling with anti-SC35 antibody since SC35 was shown to be colocalized with the RNA. The CGTAT construct which expressed the Tat RNA with an intron was used as an example to examine the RNA localization by electron microscopy. The localization of the RNA (Fig. 9 *a*) was found to correspond to a cluster with a granular appearance that also contained swirls of fibrils (Fig. 9, *b* and *c*). The structure of this cluster somewhat resembled interchromatin granule clusters seen in cells which were not transfected (for a review see Spector, 1993). However, they were larger in size and represented the active sites of transcription of the respective RNA. Therefore, while the observed RNA localization

signal appeared to be in clusters which morphologically resembled typical interchromatin granule clusters, their molecular composition may be different.

Variations in the Expression of Exogenous Templates after Transient Transfection

Throughout our transient transfection experiments, we have found a large variation in the expression levels of various constructs and the same construct among different cells or different expression loci within a single cell. A comparison of the images in Figs. 2 and 3 shows that a large portion of the localization sites of intron-less RNA or RNA with a partial intron (Fig. 2) are smaller than the

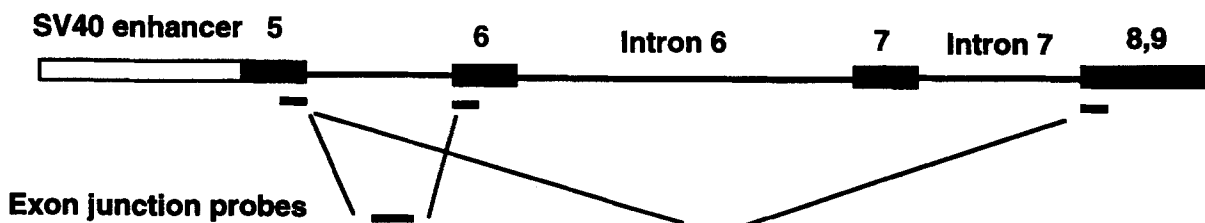


Figure 7. Diagram illustrating the design of hybridization probes for the specific detection of β -tropomyosin pre-mRNA and mature RNA. Introns 6 and 7 were used as pre-mRNA specific probes. Splice junction probes consisted of 12 nucleotides from the 3' end of an upstream exon and 12 nucleotides from the 5' end of a downstream exon. Splice junctions between exons 5 and 6 or exons 5 and 8 were used for detection of mRNA.

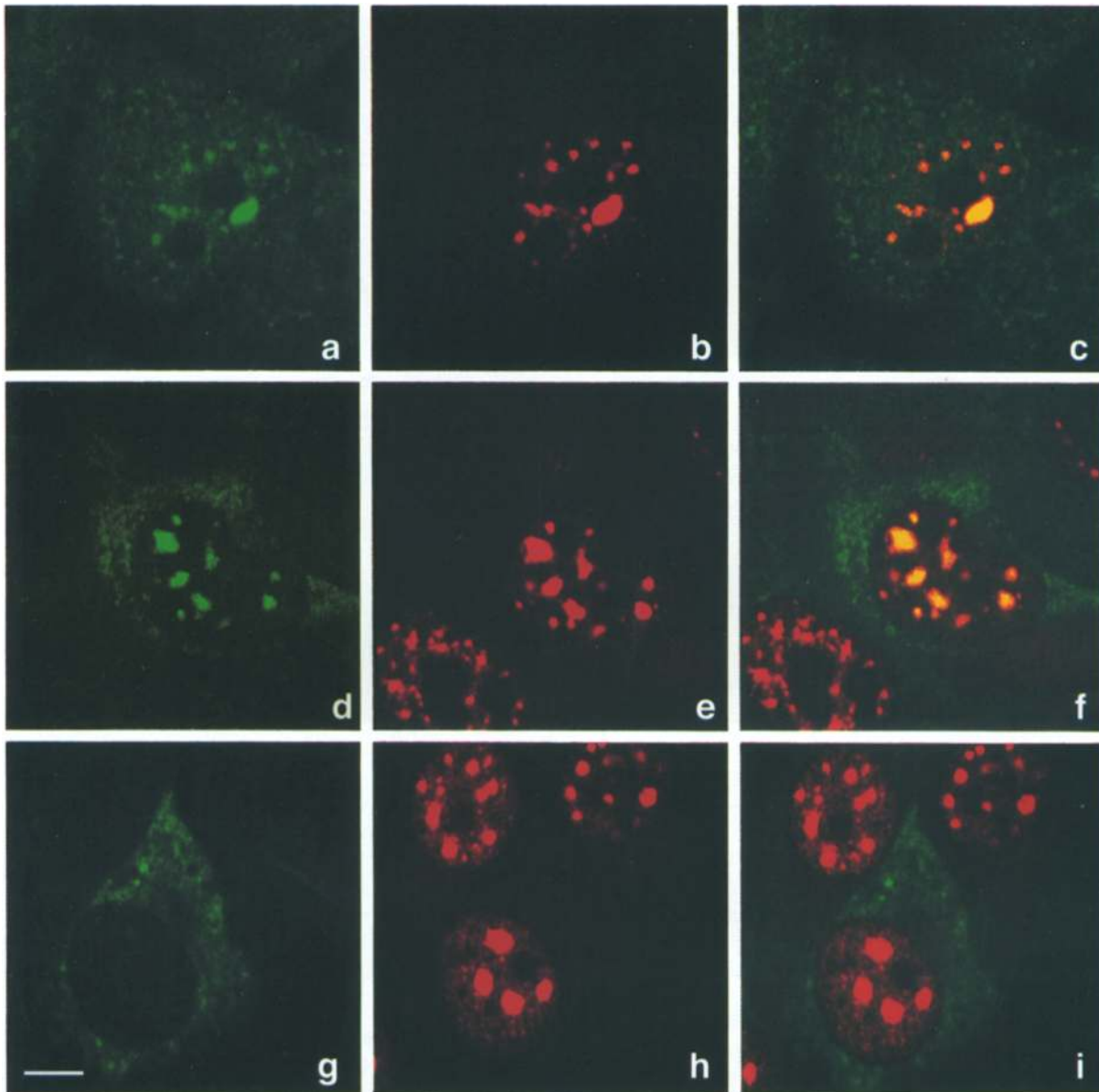


Figure 8. The localization sites of transiently expressed intron-containing RNA in HeLa cells represented the sites of pre-mRNA splicing. Detection signals of β -tropomyosin minigene mRNA (a) and pre-mRNA (b) were found to colocalize (c). In some cells, pre-mRNA was detected in the cytoplasm (d). SC35 was simultaneously detected (e). The nuclear pre-mRNA colocalized with SC35 (f). When RNA transcription was inhibited for 5 h in the presence of α -amanitin (50 μ g/ml), β -tropomyosin minigene RNA was no longer detected in the nucleus (g and i). SC35 was found in round and enlarged nuclear clusters, a pattern typically associated with α -amanitin treatment (h and i). The bar represents 10 μ m.

localization sites of intron-containing RNA (Fig. 3). Such observations suggested that the expression level of intronless RNA may be lower than that of intron-containing RNA. These observations, at the single cell level, are consistent with previous studies using S1 nuclease protection assays or nuclear run-on experiments which found that introns increase the expression of RNA transcripts (Aronow et al., 1989; Brinster et al., 1988; Buchman and Berg, 1988; Chung and Perry, 1989; Gallis et al., 1987).

The expression of intron-containing RNA also varied. Using the example of β -tropomyosin minigene expression, we have found that the expression level differed tremen-

dously from cell to cell, and from 7 h to 24 h posttransfection. At the beginning of the expression period, when cells were examined 7 h after transfection, the localization sites of the RNA tended to be smaller in size in some cells. At this stage, the distribution of the splicing factors in the same cells appeared to be in a somewhat normal speckled distribution, and, in addition, to be colocalized at the RNA expression sites (data not shown). However, at 24 h posttransfection the size of the RNA localization signal was much larger. At this stage, nearly all of the splicing factors detected in the cells colocalized with the RNA derived from the transfected templates (data not shown). Approx-

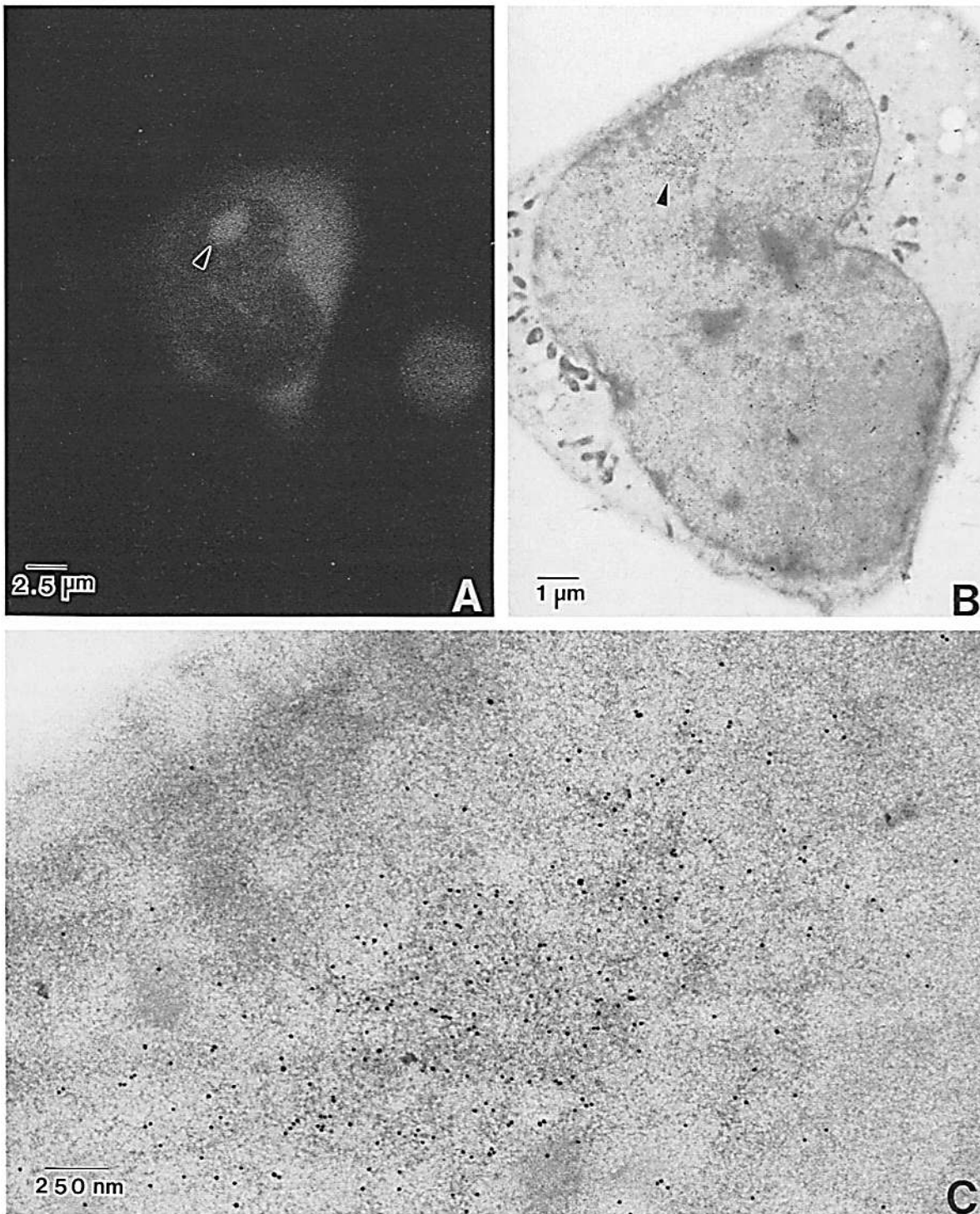


Figure 9. Electron microscopy demonstrates that the nuclear region occupied by actively transcribed intron-containing RNA consists of granular structures with swirling fibrils. After FISH, cells were processed for electron microscopy and immunolabeled with an antibody specifically recognizing SC35. The distribution of the fluorescence signal (*arrowhead* in *A*) correlated with the localization of SC35 in the same cell (*arrowhead* in *B*). EDTA regressive staining showed this region to be enriched in RNPs. Examination at higher magnification (*C*) demonstrated the granular nature of these regions reminiscent of interchromatin granule clusters in nontransfected cells.

imately 20% of these cells displayed a significantly reduced general level of endogenous cellular transcription, as compared to adjacent cells that were not transfected when examined by BrUTP incorporation. Some cells at

late stage posttransfection showed a nearly complete inhibition of endogenous transcriptional activity as well as the transcription of the exogenous template (data not shown). To avoid all of these variations, which may affect cell via-

bility, we have restricted our studies to the evaluation of cells 7 h posttransfection.

Discussion

Transcription and Pre-mRNA Splicing Are Spatially Associated

Our findings that the spatial association between nascent RNA transcripts and splicing factors is intron-dependent has demonstrated that this colocalization represents a functional interaction rather than being a random association. In addition, our observations that pre-mRNA and mRNA are colocalized at the sites of transcription support and further extend the idea that transcription and splicing are closely linked. The close association between these processes has been proposed in earlier studies that have examined nascent RNA transcripts of early *Drosophila* embryo genes (Beyer and Osheim, 1988), genes in mouse and *Drosophila* somatic cells (Fakan et al., 1986), as well as adenovirus 2 and β -actin genes in HeLa cells (Zhang et al., 1994). Our findings are also consistent with observations showing spliceosomes on nascent RNAs transcribed from the Balbiani ring genes of the dipteran *Chironomus* (Kiseleva et al., 1994), and splicing factors on the loops of lampbrush chromosomes in amphibian germinal vesicles (Wu et al., 1991).

Using a transient transfection assay, we have observed a wide range in the levels of RNA expression among nuclei and at various times after transfection. The colocalization of intron-containing RNAs with splicing factors varied from small dots to large clusters. We interpret these findings to be the result of a kinetic equilibrium among at least four parameters, the rate of transcription, the rate of splicing, the rate of dissociation of mRNA from the spliceosome, and subsequently transport away from the site of transcription. In addition, the copy number of templates at one locus may also contribute to the size of the RNA localization signal. If the rate of pre-mRNA splicing is faster than the rate of transcription, the amount of splicing factors localized at the site of transcription at any given moment will be low and may approach the sensitivity limits of the light microscope. This situation could account for the observation by Zhang et al. (1994) in which the β -actin pre-mRNA was observed to be spliced at the sites of transcription, but the splicing factors were not detected at those loci. Since pre-mRNA splicing occurred at these sites, it is unlikely that splicing factors were not present at the same nuclear regions. The simplest explanation is that the amount of splicing factors present were below the sensitivity of light microscopic detection. However, a reevaluation of the localization of β -actin RNA and splicing factors by Xing et al. (1995) has revealed a colocalization in 89% of the cases observed. If the rate of transcription of a gene is much higher than the rate of splicing or the rate of dissociation from the spliceosome, the RNA localization signal at the transcription site would be larger and concomitantly an increased number of splicing factors would be recruited to, and be present at, the sites of transcription. Ultimately, the extreme overexpression of an RNA could exhaust the available splicing factors and/or nuclear

retention factors in the nucleus. At late time points posttransfection (24–48 h), we have observed transiently expressed RNAs to leave the sites of transcription, which are presumably saturated with transcripts, and to be localized throughout the speckled pattern, at some sites that are not transcription sites (Huang, S., and D.L. Spector, unpublished data). This situation suggests that RNAs are being synthesized faster than splicing factors are being recruited to the sites of transcription. Thus, RNAs are being released from the template before being associated with splicing factors and being spliced. In addition, at these late time points posttransfection, unspliced RNA is found in the cytoplasm suggesting that pre-mRNA splicing and/or nuclear retention is a saturable process.

Splicing Factors Are Recruited to the Sites of Transcription

At some of the more abundant expression sites of the intron-containing RNAs, splicing factors and the nascent RNAs were colocalized to the same nuclear regions in large clusters which are much larger than a normal speckle. In addition, the fluorescence intensity of the splicing factor localization at these transcription sites appeared higher than at endogenous speckles elsewhere in the nucleus where these RNAs were not present. The larger and more intensely concentrated clusters of splicing factors at the sites of transcription suggested that these factors were recruited from elsewhere in the nucleus upon the initiation of transcription of intron-containing RNA. Furthermore, in the study of both transiently and stably expressed intron-containing RNAs, we have found that the shape of the RNA localization resembled the localization of the splicing factors at the same sites. These observations suggest that the recruitment of splicing factors to these sites depends upon the presence of pre-mRNA. These findings confirm earlier studies using adenoviral infection that showed splicing factors (snRNPs) (Walton et al., 1989; Jiménez-García and Spector, 1993; Pombo et al., 1994), RNA polymerase II, and hnRNP C protein (Jiménez-García and Spector, 1993) were recruited to sites of adenovirus transcription.

There are at least two different mechanisms that could explain the movement of splicing factors from storage and/or reassembly sites to the transcription sites of intron-containing RNA. First, the transcription of intron-containing RNA may trigger a signal transduction system which directs the movement of splicing factors to the sites of transcription. The triggering signal could be the newly synthesized intron-containing RNA, or the transcription apparatus itself, such as the binding of polymerase and transcription factors to the promoter. Splicing factors which are normally in excess at their sites of storage and/or reassembly would respond to the activation of transcription and move to those sites. One possible way of regulating the localization of splicing components is phosphorylation and dephosphorylation. A recently identified serine kinase, SR protein kinase 1 (SRPK1), has been shown to be involved in the phosphorylation of SR proteins (Gui et al., 1994a,b). Addition of this kinase to permeabilized cells results in the dissociation of the speckled distribution of splicing factors (Gui et al., 1994a). Alternatively, it is also possible that

there is a soluble pool of splicing components which are ready to function in the nucleoplasm; the continuously generated intron-containing RNAs would recruit those factors and deplete the soluble pool. Some of the excess splicing factors at the sites of storage and/or reassembly would subsequently be released to supplement the soluble pool so that a certain concentration of these factors can be maintained. Both of these models could explain the recruitment of pre-mRNA splicing factors to sites of active transcription. Future experiments are needed to distinguish between these or other possibilities.

Analysis of the Structural Characteristics of the Sites of Transcription of Intron-containing RNA

We have examined the sites of transcription and splicing of transiently expressed intron-containing RNA using electron microscopy. We observed a granular structure containing swirling fibrils. EDTA regressive staining (Bernhard, 1969) revealed that these regions were RNP-enriched structures. These granular structures closely resembled interchromatin granule clusters observed in nontransfected cells (Spector, 1993). However, they were much larger in size and they corresponded to sites of transcription. Classical studies have previously defined some of the functional characteristics of interchromatin granule clusters (for a review see Spector, 1993). Interchromatin granule clusters are composed of a group of 20–25 nm granules, some of which appear to be connected to each other giving the appearance of beads-on-a-string. It is not clear as to what the exact molecular composition of a single granule is and if all of the granules have the same or a different molecular composition. A variety of splicing factors including snRNPs and the SR-protein family (for a review see Spector, 1993), as well as stable poly(A)⁺ RNA (Huang et al., 1994) have been found to be enriched in interchromatin granule clusters in interphase nuclei. Since these clusters do not actively incorporate [³H]uridine after a short pulse labeling, they are not likely to represent sites of active transcription (for a review see Fakan, 1994).

Since most of the essential splicing factors including snRNPs and non-snRNP splicing factors have been found to be enriched in the interchromatin granule clusters in normal interphase nuclei, it is possible that the granular feature of the interchromatin granule clusters is due to the presence of a high concentration of splicing factors in these clusters. We interpret the observation of granular structures at the highly active transcription sites to be the result of the unusual amount of splicing factors recruited to those sites. This explanation will be further tested using a stable and inducible expression system where the expression of an intron-containing RNA can be turned on and off, and the structural organization of the region can be directly examined. Although the granular feature observed at the site of transcription of the transiently expressed intron-containing RNA morphologically resembles an interchromatin granule cluster in an interphase nucleus, the difference is that the splicing factors at these sites of transcription are actively engaged in splicing, whereas the factors in the typical interchromatin granule clusters are most likely not involved in splicing activity. Our findings do not

conflict with the previous concept that most interchromatin granule clusters are not involved in the transcription or splicing of nascent RNA transcripts.

The Speckle Concept: A Model

The relationship of the speckled distribution of splicing factors to the functions of transcription and pre-mRNA splicing has at times been controversial. Studies described above have extended and clarified our understanding of the spatial and temporal relationship between splicing factors and transcription in the mammalian cell nucleus. We provide the following model of the functional organization of the cell nucleus.

In a typical mammalian nucleus, splicing factors that are not engaged in splicing are mostly stored and/or reassembled in the larger speckles (interchromatin granule clusters). When transcription of intron-containing genes is activated, splicing factors are recruited to the sites of transcription. The amount of splicing factors recruited to the sites of transcription depends upon the kinetic equilibrium of the transcription level of the gene, the efficiency of RNA splicing, dissociation of the mRNA from the splicing complex, and the transport of the mRNA away from the transcription sites. When RNAs are expressed at low or moderate levels, the amount of splicing factors recruited to the sites of transcription is low and could be below the sensitivity of light microscopic detection. Such transcription sites are visualized as perichromatin fibrils at the electron microscopic level which are diffusely distributed throughout the nucleus and are present on the surface of some of the interchromatin granule clusters; this would be the case for most cellular genes. However, when a gene is expressed at a very high level, a large number of splicing factors are recruited to the site of transcription resulting in a granular appearance which closely resembles, but is functionally different from a typical interchromatin granule cluster. Such is the case when one examines the sites of transcription of transiently expressed templates or highly expressed endogenous RNAs such as collagen I α 1 subunit (Xing et al., 1995; Huang, S., and D.L. Spector, unpublished data) in young fibroblasts. The intron-dependent recruitment of splicing factors to the sites of active transcription suggests that the movement of splicing factors is highly regulated. Therefore, we propose that the organization of splicing factors is dynamic and reflects the transcriptional activity of the cells.

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