Dynamic relocation of transcription and splicing factors dependent upon transcriptional activity

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Recent interest in understanding the spatial organization of gene expression has focused attention on nuclear structures known as speckles or interchromatin granule clusters (IGCs) revealed by immunofluorescence or electron microscopy. Staining of nuclear factors involved in pre-mRNA splicing or, more recently, transcription, reveals 20–40 speckles per nucleus, resulting in the intriguing suggestion that speckles are nuclear sites of transcription and processing. In contrast, other investigations have observed transcription in other areas of the nucleus. In this study, we have examined the localization of active transcription as detected by uridine incorporation and recently developed RNA polymerase II antibodies, and compared this pattern with that of known splicing and polyadenylation factors. Our results indicate that in actively transcribing cells, transcription and splicing factors are dispersed throughout the nucleus with abundant sites of preferred localization. In contrast, in poorly transcribing cells, polymerase II and splicing factors localize to speckles. In nuclei inactivated for transcription by drugs or heat shock, the speckle type of co-localization is accentuated. These observations suggest that bulk transcription and splicing occur throughout the nucleus during periods of active transcription; and that factors involved in these two processes re-locate to minimal speckle domains during periods of inactive transcription.

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Introduction

In recent years, a number of studies have addressed the spatial organization of gene expression within mammalian nuclei. Key to this endeavor have been experiments localizing polymerase II-derived nascent transcripts and the factors that transcribe, splice and polyadenylate these RNAs. A number of antibodies specific for elements of the transcription or pre-mRNA processing machinery have been used to reveal subnuclear structures associated with pre-mRNA (reviewed in Spector, 1993; Fakan, 1994; Moen, *et al.*, 1995; van Driel *et al.*, 1995). One particularly striking staining pattern is that observed using an antibody raised against the conserved splicing factor, SC35. This antibody brightly stains nuclei from a wide variety of

tissue culture cells in a pattern of localized staining termed ‘speckles’ (Fu and Maniatis, 1990; Spector *et al.*, 1991). Other splicing-related factors were also localized to speckles (Spector, 1984, 1990; Nyman *et al.*, 1986; Carmo-Fonseca *et al.*, 1992; Huang and Spector, 1992; Krause *et al.*, 1994; for reviews, see Spector, 1993; van Driel *et al.*, 1995). Cultured mammalian cells commonly contain 20–40 speckles, raising the intriguing possibility that RNA processing is restricted to minimal domains within mammalian nuclei. Such a hypothesis immediately suggested either that many pre-mRNAs should be synthesized within the vicinity of speckles or that nascent pre-mRNAs must be transported to the speckles for processing.

Experiments to address the possibility that speckles represent sites of transcription or processing of pre-mRNA have utilized localization of either bulk or individual transcripts. Use of oligo(dT) probes to detect bulk polyadenylated RNA revealed a speckled localization (Carter *et al.*, 1991, 1993). Several identified unique pre-mRNAs have also been localized to speckles defined by staining with the anti-SC35 antibody (Huang and Spector, 1991; Lawrence *et al.*, 1993; Xing *et al.*, 1993, 1995; reviewed in Moen *et al.*, 1995). Speckled localization was especially prominent for microinjected RNAs or transcripts produced from transfected DNAs (Wang *et al.*, 1991; Huang and Spector, 1996). Such data suggested that the speckled pattern reflected localized accumulation of active splicing and transcription factors.

An alternative proposal has suggested speckled nuclear structures as storage sites of splicing and transcription factors distal from the sites of bulk transcription occurring throughout the nucleoplasm and associated with chromatin (reviewed in Spector, 1993; van Driel *et al.*, 1995). Supporting evidence for this proposal came from early electron microscopy studies in which bulk transcription was localized by short pulses of [³H]uridine incorporation. Nascent RNA was detected within the perichromatin fibrils that reside between the interchromatin granule clusters (IGCs) with little accumulation at the IGCs, or speckles (reviewed in Fakan and Puvion, 1980; Fakan, 1994; Hendzel and Bazett-Jones, 1995). Subsequent *in situ* nucleotide incorporation experiments using fluorescence microscopy have confirmed this observation via the detection of transcription at many sites within the nucleus other than the speckles (Jackson *et al.*, 1993; Wansink *et al.*, 1993). Furthermore, multiple identified pre-mRNAs have been localized to regions of the nucleoplasm between the speckles (Zhang *et al.*, 1994). These results raised questions about speckles being the sites of pre-mRNA generation and subsequent splicing. Such incorporation studies, however, are compromised by the inherent difficulty in ascribing bulk transcription to pol II-driven pre-mRNA synthesis (Moen *et al.*, 1995).

Determining the function in transcription and splicing

of speckles is an important issue because of the ramifications that alternate interpretations have on the spatial relationship between transcription and RNA processing within the nucleus. If transcription and processing are coupled, as studies in *Drosophila* indicate (Beyer *et al.*, 1988), then it is difficult to visualize how all of the active transcription sites in the average nucleus can be effectively coupled to 20–40 speckles containing processing factors. It also becomes difficult to explain why so much transcription occurs distal to speckles when *in situ* incorporation experiments are performed. If transcription and splicing are spatially uncoupled within the nucleus, it is difficult to envisage how extremely large pre-mRNAs can be made as a unit and then transported within the nucleus to speckle-type processing centers. Thus, it is difficult to reconcile observations that indicate disperse transcription centers and localized processing centers. Given the striking visual observation that many factors involved in splicing can be found in speckles, it becomes imperative to ascertain if speckles are indeed the sites of active pre-mRNA production and/or processing.

Recently, two antibodies directed toward RNA polymerase II were developed (Bregman *et al.*, 1995). One of these antibodies recognizes pol II hyperphosphorylated within the C-terminal domain (CTD) of the large subunit, whereas the other recognizes hyper-, hypo- and intermediately phosphorylated forms of the CTD. In this study, we show that both pol II antibodies can recognize transcriptionally engaged polymerase molecules, indicating that both antibodies can be used to detect sites of active transcription within the nuclei of mammalian cells. Using these antibodies and other antibodies specific to RNA processing factors, we have observed that transcription and RNA processing overlap within a broad meshwork occupying the non-nucleolar portion of the nucleus with hundreds of ‘dots’ of preferred localization overlaying the network. Observation of the meshwork was dependent on the ability to detect active transcription by bromo-uridine triphosphate (BrU) incorporation. In cells in which BrU incorporation was abundant, BrU incorporation, the large subunit of polymerase II, and splicing and polyadenylation factors were broadly located within the nucleoplasm and did not concentrate at speckles.

In contrast, cells that poorly incorporated BrU demonstrated a strikingly different pattern in that both pol II antibodies and splicing factor antibodies co-localized to classic speckles, suggesting a redistribution of transcription and splicing factors during periods of inactive transcription. A similar speckled localization of pol II was observed when transcription was experimentally arrested with either drugs or heat shock, supporting such a redistribution. Therefore, our data suggest that active transcription and splicing occur throughout the nucleoplasm. Furthermore, they support an alternative definition of speckles as dynamic locales within the nucleoplasm in which transcription and processing factors localize between rounds of active pre-mRNA synthesis, perhaps for some form of recycling and reactivation. Our data also suggest that very active transcripts, such as those previously localized to speckles, may be found preferentially in, or adjacent to, speckles because they provide a local high concentration of processing factors following recycling.

Results

Monoclonal antibodies H5 and H14 recognize transcriptionally engaged polymerase II molecules

Monoclonal antibodies reactive for phosphorylated forms of mammalian polymerase II have recently been developed (Bregman *et al.*, 1995). One of these antibodies, mAb H5, is specific for a hyperphosphorylated form of the large subunit of pol II (Ilo); a second antibody, mAb H14, recognizes various forms of the phosphorylated pol II large subunit. A population of non-transcribing polymerase molecules that are phosphorylated on the CTD has been detected by both H5 and H14 (Bregman *et al.*, 1995; Kim *et al.*, 1997). However, phosphorylation of the CTD takes place concomitant with the initiation of transcription (reviewed by Dahmus, 1995); consequently, we asked if mAb H5 and H14 recognize phospho-epitopes on actively transcribing polymerase molecules. For this purpose, we used an *in situ* photoaffinity labeling technique (Bartholomew *et al.*, 1986). Briefly, nuclear run-on transcription was performed with isolated HeLa cell nuclei in the presence of [α - 32 P]CTP (labeling nucleotide), 4-thio-UTP (photoactive RNA–protein cross-linking nucleotide) and 3'-O-methyl GTP (chain-terminating nucleotide). Then nuclei were exposed to UV light ($\lambda_{\text{max}} = 310 \text{ nm}$) to induce covalent cross-linking of the radiolabeled RNAs to proteins. The nuclei were extracted after digestion with RNase to trim excess RNA from the proteins, and the extract was used immediately for immunoprecipitation with mAbs H14 and H5.

The results indicated that, in the absence of the transcription inhibitor α -amanitin, mAbs H5 and H14 immunoprecipitated three distinct [α - 32 P]CTP-radiolabeled polymerase II subunits: Ilo (~240 kDa), Ila (~220 kDa) and Ilc (140 kDa) (Figure 1A). mAb H5, which recognizes a hyperphosphorylated form(s) of polymerase II (Bregman *et al.*, 1995), immunoprecipitated predominantly [α - 32 P]CTP-radiolabeled Pol Ilo. Trace [α - 32 P]CTP-radiolabeled Pol Ila was also present (Figure 1A, lane 1). mAb H14, which recognizes multiple phosphorylated forms of polymerase, immunoprecipitated [α - 32 P]CTP-radiolabeled Pol Ilo as well as a significant amount of Pol Ila (Figure 1A, lane 4). In the presence of α -amanitin, radiolabeled RNA was not detected on immunoprecipitated polymerase molecules. Thus, mAbs H5 and H14 can recognize a population of phosphorylated polymerase molecules that is transcriptionally engaged in the *in situ* nuclear run-on assay.

Polymerase II localizes to many sites within the nucleoplasm

Next, we wanted to know the nuclear localization of pol II that is actively engaged in transcription. mAb H5 and mAb H14 have been shown to stain mammalian cells broadly (Bregman *et al.*, 1995). Figure 1B and C indicates a staining of human ovarian cancer cells (OV-MZ-15; Möbus *et al.*, 1994) with antibodies mAb H5 (Figure 1B) and mAb H14 (Figure 1C). Broad staining of the entire non-nucleolar portion of the nucleus was observed, suggesting a wide distribution of pol II and, by inference, of transcription throughout the nucleoplasm. In this communication, we refer to this nucleoplasm-wide staining as a meshwork because at higher resolution of the image,

Fig. 1. (A) Transcriptionally active polymerase molecules are recognized by mAbs H5 and H14. 32 P-labeled RNA was covalently cross-linked to transcriptionally active polymerase molecules in isolated nuclei (see Materials and methods). A nuclear extract prepared from these nuclei was used for immunoprecipitation with mAb H5 (specific for the hyperphosphorylated pol II) and mAb H14 (specific for multiple phosphorylated forms of pol II), mAb H22 (control IgM) and mAb M2 (control IgG). Ilo, hyperphosphorylated largest subunit of RNA polymerase II; Ila, hypophosphorylated largest subunit of RNA polymerase II; Ilc, second largest subunit of RNA polymerase II. Numbers at left margin indicate apparent molecular weights in kDa. (B and C) Nuclear localization of pol II. OV-MZ-15 cells were immunostained with mAb H5 (B) or mAb H14 (C). The broad staining of the non-nucleolar portion of the nucleus is referred to in this communication as a meshwork. Overlaying this meshwork were multiple discrete sites of preferred staining we have termed 'dots'. Bar = 5 μ m.

staining was not uniformly diffuse, but instead appeared as a very dense fibrogranulose structure. Overlaying the meshwork were multiple discrete sites of preferred staining distinguishable from a classic speckle staining pattern. These discrete sites were very numerous in several tested cell lines, with an average number of 100–200 per cell in a given focal plane (suggesting up to 1000 per nucleus) and were observed throughout the entire nucleoplasm (with the exception of nucleoli). Therefore, preferred sites of staining for pol II were noticeably more numerous per nucleus than the number of speckles per nucleus. Most sites detected by pol II-specific antibodies were also smaller in appearance than classic speckles. To distinguish linguistically this latter pattern of pol II localization from that classically referred to as speckles, we have termed these multiple sites of pol II localization nuclear 'dots'.

Polymerase II localization pattern is distinct from speckles

Speckles are frequently experimentally defined by staining with antibodies specific for splicing factors. One such factor, SC35, has been prominently localized to speckles (Fu and Maniatis, 1990; Spector *et al.*, 1991). Figure 2A–C compares the staining pattern of cells stained with both pol II- and SC35-specific antibodies. Both antibodies stained broadly throughout the nucleoplasm in a meshwork pattern. Although the meshwork was much more visible with the pol II antibody in optical sections, the two patterns of staining overlapped extensively, suggesting that a portion of the SC35 appears to be localized in the vicinity of polymerase. Superimposed on this meshwork

pattern were the prominently staining speckles of SC35 (Figure 2A, green) or the nuclear dots of pol II (Figure 2B, red). A few of the speckles overlapped with the 'dots' of polymerase II (overlay in Figure 2C); for the most part, however, the dots of preferred pol II localization and the SC35-defined speckles did not co-localize. Our result suggests that the bulk of polymerase II is not localized to speckles and raises questions about models that propose speckles as the sites of active transcription and RNA processing.

Uridine incorporation and pol II overlap both in a meshwork throughout the nucleoplasm and in many preferred sites

To confirm that the pattern of staining detected with pol II-specific antibodies represented sites of transcription, double-label experiments were performed in which the pattern of pol II staining using antibody mAb H5 was compared with sites of UTP incorporation during an *in situ* transcriptional run-on experiment (Figure 2D–I). Nascent transcripts were labeled with BrU and visually detected via the use of anti-BrU antibodies. Human CaSki (Figure 2D–F) and OV-MZ-15 (Figure 2G–I) cells were used for this experiment.

For CaSki cells, two patterns of staining were observed that are represented by the two cells shown in Figure 2D–F. One pattern was seen in 60–80% of the cells and is represented by the top cell in Figure 2D. In these cells, nascent RNA as detected by confocal microscopy was localized throughout the nucleus in a pattern very similar to that of the pol II staining shown in Figure 2E (i.e. a

Fig. 2. Localization of pol II, BrU incorporation, and the splicing factor SC35. (A–C) Localization of pol II relative to the splicing factor SC35 by confocal microscopy. Immunofluorescence staining of SC35 (A, green) was compared with that of pol II as detected by the mAb H5 antibody (B, red; overlay in C). (D–L) Localization of nascent RNA relative to pol II by confocal microscopy. *In situ* RNA synthesis was localized by incorporation of BrUTP for 30 min and detection of incorporated BrU via the use of an antibody directed against BrU (D, G and J, green). Pol II was localized in the same cells by immunofluorescence staining with the mAb H5 (E, H and K, red). Co-localization of antibodies would appear yellow in overlaid panels (F, I and L). (D–F) Double localization in CaSki cells. Nuclei with appreciable BrUTP incorporation (top) and minimal BrUTP incorporation restricted to the nucleoli (bottom) are shown. (G–I) Double staining in a second human cell line OV-MZ-15. Note that in some double-stained foci of both cell lines, staining of pol II and BrU overlap but are not exactly the same size, shape, or intensity. (J–L) Arresting transcription causes a redistribution of polymerase II. Pol II-directed transcription was inhibited by treatment of CaSki cells with 1 mg/ml α -amanitin during an *in situ* transcription experiment. BrU incorporation was severely curtailed and bright staining was limited to nucleoli (J, green). Pol II staining was highly concentrated in speckle-like domains (K, red; overlay in L). Bar = 1 μ m.

nucleoplasm-wide meshwork overlaid with many sites of preferred staining).

OV-MZ-15 cells demonstrated localization patterns of

pol II and BrU incorporation similar to that of CaSki cells (Figure 2G–I). Both pol II and nucleotide incorporation occurred throughout the nucleoplasm in a complicated

pattern. The merged views (Figure 2F and I) demonstrated considerable overlap of the two staining patterns (yellow) with the exclusion of nucleoli (marked as arrows). Because immunofluorescence can only provide an approximation of the actual extent of co-localization of two antigens with immunofluorescence patterns as complex as those in Figure 2D–I, a conservative interpretation of these patterns suggests that both pol II and transcription are broadly dispersed throughout the nucleoplasm in a particulate, non-speckled pattern.

The underlying reason for both a meshwork and ‘dotted’ distribution of both pol II and BrUTP incorporation is unclear. Given the limits in resolution of light microscopy, the meshwork pattern could represent the transcription of single, moderately transcribed genes, whereas the dots could represent either clusters of genes or individual high-activity transcription units. Regardless of their origin, the similar appearance and location of both incorporation and polymerase suggests that both regions represent active sites of pol II-directed transcription and that both are clearly distinguishable from speckles.

Close examination of multiple double-labeling experiments indicated that the discrete sites of pol II and BrU staining in cells demonstrating nuclei-wide BrU incorporation were not absolutely coincident. The size, shape or intensity in some of overlapping domains are not exactly the same. In particular, it was usually possible to observe a subfraction of pol II staining that had little associated BrU incorporation which presumably represented the subpopulation of pol II that was not actively engaged in the transcription (Kim *et al.*, 1997). Moreover, the loci of nucleoplasmic BrU incorporation demonstrated considerable overlapping pol II staining.

In addition to H5, mAb H14, which recognizes various phosphorylated forms of the pol II large subunit, was also used in co-localization of pol II and its transcripts. In both CaSki and OV-MZ-15 cells, H14 staining appeared identical to H5 pattern as a meshwork overlaid with discrete nuclear dots which overlapped with BrU staining, suggesting that different phosphorylated forms of pol II may exist in transcription domains. This result agrees with the results shown in Figure 1A that both antibodies can recognize transcriptionally engaged polymerase molecules.

Pol II localizes to speckles in cells not actively involved in transcription

A second pattern of *in situ* transcription was observed with CaSki, but not OV-MZ-15 cells, and is represented by the bottom cell in Figure 2D–F. This cell, representing 20–40% of the population, stained minimally with the anti-BrU antibody, indicating a poor incorporation of BrU. Incorporation in these cells was restricted to nucleoli when detected with phase-contrast microscopy (data not shown). In this second subpopulation of cells, pol II staining was concentrated into minimal domains that resembled speckles. A speckled distribution of pol II has been reported previously for MDCK cells (Bregman *et al.*, 1995). In this latter study, speckles were preferentially seen in cells within the early G₁ or late G₂ phases of the cell cycle, in ~20% of the cells within an unsynchronized cell population.

The data in Figure 2D–F indicated that the pattern of pol II localization depended on the distribution of nascent

transcripts, those cells that demonstrated active, nucleoplasm-wide transcription demonstrated nucleoplasm-wide pol II localization, suggesting that in these cells the staining pattern of pol II-specific antibodies represents sites of transcription of pol II transcribed genes. In contrast, the ‘speckle’ staining pattern for pol II was only observed in cells in which transcription was limited to the nucleoli, suggesting an alteration in localization of pol II, depending on the ability to detect numerous nascent transcripts. It should be noted that although mAb H5 recognizes hyperphosphorylated polymerase II, it has been shown to be capable of detecting pol II in cells arrested for transcription immediately before or after mitosis or with drug treatment (Bregman *et al.*, 1995), indicating that the phosphorylated CTD epitope is still present under these conditions. In agreement with this interpretation, cells with and without appreciable BrU incorporation had approximately equal staining with mAb H5 (compare the two cells in Figure 2E), suggesting maintenance of the phosphorylated pol II epitope detected by this antibody in cells not actively incorporating BrU.

We interpret the lack of nucleoplasm-wide pol II staining and BrU incorporation in the minor cell population to reflect minimal pol II-directed transcription in these cells. Alternative interpretations, including lack of permeability to both anti-pol II antibodies and nucleotide analogs, or inaccessibility of antigens to antibodies, seem unlikely because of the ability of pol II actively to decorate speckles in these cells as well as the ability to detect nucleotide incorporation into nucleoli in the same population.

In vitro splicing has recently been shown to be inhibited if all uridines (U) within the utilized precursor RNA were replaced with BrU, presumably due to a strict requirement for U in consensus splicing sequences (Wansink *et al.*, 1994). To address possible problems in factor localization arising from the use of a nucleotide analog, we compared the staining patterns of BrU, pol II and SC35 in CaSki and OV-MZ-15 cells during *in situ* transcription experiments using mixtures of UTP and BrUTP in various ratios, or in the total absence of BrUTP. The BrU staining pattern remained the same when the ratio of UTP:BrUTP was varied over a 4-fold range (data not shown), indicating minimal impact on the sites of transcription as BrUTP was added. More importantly, the pattern of pol II and SC35 staining remained the same when cells were transcribed in the absence or presence of BrUTP (data not shown). Thus, the distribution patterns of pol II and SC35 were not affected by the presence of BrU during an *in situ* transcription experiment.

Inactivation of transcription localizes pol II to speckles

The above experiments suggested a correlation between transcriptional state and the localization of polymerase II within the nucleoplasm. To better correlate the two, experiments were performed in which transcription was halted by either treatment with α -amanitin or heat shock (Figures 2J–L and 3, respectively). When α -amanitin was added to the *in situ* transcription of CaSki cells at a concentration sufficient to inhibit only polymerase II, BrU incorporation was severely curtailed in the entire population and bright staining was limited to nucleoli (Figure 2J). In all cells, pol II staining was highly

Fig. 3. Heat shock causes a redistribution of polymerase II in MDCK cells. When cells were fixed and then permeabilized with non-ionic detergent, mAb H5 normally stained a meshwork, non-speckled pattern (A). Cells subjected to a heat shock of 45°C for 1 h, and stained with the same antibody showed a speckled pattern (B). After recovery at 37°C for 1 h, the speckled distribution of pol II disappeared and the meshwork staining pattern was restored (C); recovery at 0°C maintained pol II staining as a speckled pattern (D). Bar = 10 μ m.

concentrated in a minimal number of centers, to produce a pattern closely resembling that of speckles (Figure 2K and L). Thus, when pol II activity was inhibited and the synthesis of pre-mRNA arrested, pol II staining was highly localized.

Heat shock also inhibits pre-mRNA production. When MDCK cells were subjected to a heat shock of 45°C for 1 h, pol II distribution as monitored by staining with the mAb H5 antibody was concentrated into speckles as distinguished from the nucleoplasmic meshwork staining in cells at normal temperature (compare Figure 3B with Figure 3A). If permitted to recover at 37°C for 1 h, the speckled distribution of pol II disappeared and the normal meshwork staining pattern was restored. Recovery of pre-heat shock distribution of pol II required normal temperature; recovery at 0°C caused maintenance of pol II staining in a speckled pattern (Figure 3C and D, respectively). As with α -amanitin treatment, pol II distribution was throughout the nucleoplasm under conditions permitting transcription, but was more localized into a speckle-like pattern when pre-mRNA production was inhibited. The reversible reorganization of pol II upon heat shock suggests a possible cycling of polymerase between speckles and more widely distributed sites of transcription.

To confirm that the minimal staining domains of pol II detected when transcription was arrested actually were speckles, the staining pattern of pol II and SC35 were

compared in CaSki cells under conditions of *in situ* transcription (Figure 4). As before, pol II staining was observed in different patterns in subpopulations of cells: a broad meshwork was observed in a majority of cells with pronounced 'speckles' in a minority of cells (speckles marked with arrows in Figure 4A). In addition, there were cells in the population that demonstrated an intermediate pattern of pol II distribution. The speckles of pol II correlated well with the speckles of SC35 (Figure 4B). Interestingly, SC35-detected speckles were most pronounced in those cells demonstrating pol II speckles. In those cells in which pol II was more dispersed to the meshwork and nuclear dots, the SC35 speckles were less pronounced, suggesting that SC35, like pol II, may redistribute dynamically within nuclei depending on transcription (see also Figure 6).

Polyadenylation and splicing factors overlap with sites of transcription

Similar appearance of *in situ* BrU incorporation and polymerase II within both a broad meshwork and dots throughout the nucleoplasm permitted us to re-address the question of co-localization of transcription and RNA processing with an emphasis on the non-speckled portion of the nucleus. Figures 5 and 6 examine the localization of transcription as measured by BrU incorporation versus the localization of the polyadenylation factor CstF and the arginine-serine-rich (SR) family of splicing factors.

Fig. 4. Dynamic localization of pol II and the splicing factor SC35 dependent upon transcription. Localization of pol II (**A**) and SC35 (**B**) in CaSki cells was compared in an *in situ* transcription experiment. A range of pol II distribution similar to that in Figure 2E is shown (**A**), ranging from an utterly dispersed pattern (left three cells) to a pattern containing both dots and speckles (arrows) overlaying the meshwork staining. SC35 localization (**B**) also varied but to a lesser degree, with sharper speckles in cells with a more speckled pol II distribution (right) and appearance of background meshwork staining in cells with more distributed pol II (left). Bar = 5 μ m.

CstF is a highly conserved heterotrimer involved in the polyadenylation of pol II-transcribed pre-mRNA. Previous studies had indicated that antibodies to CstF broadly stain nuclei (Takagaki *et al.*, 1990). Recently, cleavage factors CstF and CPSF were also shown to be enriched in a few 'cleavage bodies', some of which contained newly synthesized RNA (Schul *et al.*, 1996). When used to stain OV-MZ-15 cells in an *in situ* BrU incorporation study, antibodies to BrU and the 64 kDa subunit of CstF appeared to block each other in many cells, i.e. if a cell was significantly stained by one antibody, the intensity of the alternate staining was too weak to record. This blocking effect strongly suggested co-localization of epitopes in nascent RNA and CstF within the nucleus. A portion of stained cells showed less blocking and permitted visualization of CstF in a broad meshwork pattern similar to the pattern of BrU incorporation (Figure 5A and B,

respectively), suggesting that polyadenylation factors and polymerase II are localized in the same or adjacent regions of the nucleus. It should be noted, however, that certain 'dots' accentuated in the BrU incorporation stained only poorly with the anti-CstF antibody, suggesting either a population of pol II-directed transcripts not requiring polyadenylation, such as histone pre-mRNA, or partial masking of one antibody for the other. Cumulatively, in addition to antibody blocking, CstF staining and BrU incorporation overlap broadly over the nuclear meshwork.

Staining with antibody 104, which detects the major members of the mammalian SR family of splicing factors (Zahler *et al.*, 1992), also stained nuclei broadly in those cells that actively incorporated BrU (Figure 6A–C). A portion of the broad staining appeared to overlap with nascent RNA; a portion of the BrU incorporation was not associated with appreciable mAb 104 staining. Thus, the

Fig. 5. The polyadenylation factor CstF broadly overlaps *in situ* transcription. BrU incorporation (A) in OV-MZ-15 cells was compared with staining with an antibody specific for the 64 kDa subunit of CstF (B). Extensive overlap between the two antibodies was seen in both the background meshwork and the dots. There was an inverse correlation between the ability to detect BrU incorporation and the intensity of staining with CstF antibodies, suggesting a partial blocking of staining of CstF with the BrU antibody in these cells (marked with asterisks). In order to demonstrate the weak CstF staining due to the partial blocking of anti-BrU antibody, (B) was overexposed during photo processing. Bar = 5 μ m.

SR proteins did not seem to be as prevalently associated with pol II transcription units as did the polyadenylation factor CstF, consistent with a uniform requirement for CstF in the generation of polyadenylated RNA and a more specialized requirement for the major SR proteins family members detected by mAb 104.

As with the pol II antibodies, staining of splicing factors in those cells that actively incorporated BrU was visually different than staining in cells that poorly incorporated BrU. In these latter cells, the SR proteins detected by mAb 104 were highly localized into minimal domains of speckles. Thus, like pol II, the localization of SR proteins within the nucleoplasm was related to the ability of individual cells to demonstrate appreciable BrU incorporation.

The pattern of staining observed with the anti-SC35 antibody was also compared with the pattern of BrU incorporation in a double-label experiment (Figure 6D–F). Similar to the mAb 104 staining, the staining pattern of this individual SR protein varied from dispersed throughout the nucleoplasm (Figure 6E, top cell) to concentrated within minimal speckle domains (Figure 6E, bottom two cells). BrU incorporation was maximal in cells showing a dispersed SC35 pattern, and minimal in cells demonstrating a concentrated, speckle pattern (Figure 6D).

In cells demonstrating the more classical speckle pattern of SC35 staining, the limited BrU incorporation occurring did not co-localize with the speckles (Figure 6F). Dispersed and apparent overlap of BrU incorporation and SC35 increased as the level of overall incorporation of BrU increased, suggesting that at least a portion of the SC35 is localized throughout the nucleoplasm when BrU incorporation and transcription is occurring maximally. The overlap of SC35 staining and BrU incorporation was not as pervasive as the overlap of pol II and BrU incorporation, suggesting that SC35 is not associated with all nucleoplasmic transcripts.

Discussion

Localization of transcription within mammalian nuclei

Recently, it has become possible to localize RNA polymerase II in somatic nuclei at the level of light microscopy via the use of monoclonal antibodies H5 and H14 directed against the CTD of the large subunit (Bregman *et al.*, 1995; Kim *et al.*, 1997). Applied to several lines of cultured mammalian cells, these antibodies detected pol II throughout the nucleoplasm in a broad meshwork with several hundred sites of preferred localization. This pattern suggests that Pol II is distributed broadly in the nucleoplasm and, by inference, that pol II-directed transcription of pre-mRNA occurs throughout the nucleoplasm. Such an interpretation was substantiated via the use of *in situ* incorporation of nucleotide analogs to detect active transcription. The nuclear distribution patterns of both pol II and *in situ* nucleotide incorporation were highly complex, with both antigens distributed in a meshwork pattern that appeared fibrous under high magnification. Although co-localization of complex patterns is hard to determine unambiguously via light microscopy, the patterns of pol II and BrU incorporation appeared to co-mingle throughout the nucleoplasm, suggesting that both patterns are indicative of pol II transcription of pre-mRNA and that such transcription is not restricted to minimal domains within mammalian nuclei.

When transcription was arrested by either drugs or heat shock, polymerase II distribution altered such that it could no longer be detected as an intensive meshwork throughout the nucleoplasm and instead appeared in minimal domains corresponding to speckles. These results suggest a dynamic situation in which polymerase II is recruited to sites of active transcription located throughout the nucleoplasm and relocates during inactive transcription to minimal domains. In this view of nuclear pre-mRNA generation, speckles would be considered as temporary storage or

Fig. 6. The localization of SR proteins is dependent on *in situ* transcription activity. BrU incorporation (A and D, green) was compared with staining with mAb 104 which recognizes a family of SR proteins (B, red) or the antibody which recognizes the SR protein SC35 (E, red). Co-localization of the nascent RNA (A and D) and SR proteins (B and E) would appear yellow in (C) and (F). For both co-localizations, a field of cells is shown that varies in the efficiency of *in situ* transcription varying from highly active (top) to minimally active with label only in the nucleoli (bottom). SR localization by either 104 or SC35 staining varied from relatively dispersed throughout the meshwork and dots (top) to highly speckled (bottom). In addition, not all sites of BrU incorporation stained well with SR antibodies. Bar = 5 μ m.

perhaps recycling centers for multiple factors required for mRNA biogenesis.

Evidence that pol II could be detected in a speckled pattern has been reported previously (Bregman *et al.*, 1995). Pol II was detected in speckles most predominantly during early G₁ and late G₂ stages of the cell cycle in MDCK cells, suggesting that pol II localization was concentrated in speckles during those parts of the cell cycle associated with minimal transcription. Here, we saw pol II localization to a speckle pattern in a minor population of CaSki cells during an *in situ* transcription experiment. This population of cells incorporated BrUTP only within nucleoli, suggesting that they were not actively engaged

in pol II-directed transcription during the labeling interval. Observation of this population of pol II localized to speckles was cell line-specific; OV-MZ-15 cells, a line of human cervical carcinoma cells, for example, did not yield a speckle pattern unless transcription was deliberately blocked. Cumulatively, the observations from experiments localizing polymerase II and sites of BrU incorporation indicate that neither are localized to speckles when transcription is maximal, but that pol II can be seen in such minimal domains under natural or induced conditions when transcription is restricted.

The C-terminal domain of polymerase II contains all known phosphorylation sites in the subunit (reviewed

in Corden, 1993; Dahmus, 1995). Two pol II-directed antibodies used in this study, H5 and H14, are directed against hyperphosphorylated or various forms of phosphorylated pol II, respectively. In experiments using immunoprecipitation of nascent *in vivo* RNA (Figure 1) and *in situ* BrU incorporation, both H5 and H14 antibodies efficiently recognize newly transcribed RNA. No significant difference was observed in their abilities to bind transcriptionally engaged polymerases, indicating the involvement of different forms of phosphorylated pol II during transcription. These results also imply that the level of phosphorylation may not be well correlated with transcriptional activity *per se*, although phosphorylation occurs concomitant with the initiation of transcription and elongating pol II has been shown to be hyperphosphorylated (Laybourn and Dahmus, 1990; Kang and Dahmus, 1993; Weeks *et al.*, 1993).

Co-localization of transcription and splicing

Elegant electron microscopy of *Drosophila* salivary glands has suggested that transcription and processing are coincident with 5'-proximal introns being recognized and processed before 3'-proximal regions of a pre-mRNA have even been synthesized (Beyer *et al.*, 1988). If transcription and processing are coincident, co-localization of transcription and processing factors would be predicted. Here, we show that BrU incorporation, pol II localization and localization of one polyadenylation factor (CstF) and a family of splicing factors (the SR proteins detected by mAbs 104 and SC35) overlap throughout the nucleoplasm in a pattern of a nucleoplasmic meshwork with many sites of preferred staining that is distinguishable from speckles. This meshwork pattern of nascent RNA staining has not been the focus of previous studies because of an interest in the more visually prominent domains represented by speckles. Broad meshwork staining, however, has been noticed in studies of the distribution patterns of a number of processing factors other than SC35 (Takagaki *et al.*, 1990; Carmo-Fonseca *et al.*, 1991a,b, 1992; Jenny *et al.*, 1994; Krause *et al.*, 1994).

Our results indicate that splicing factors are located throughout the nucleoplasm in the same area in which transcription by pol II is occurring. Like the localization of polymerase II, the localization of splicing factors was dependent upon the transcriptional activity of the cell being examined. When BrU incorporation was high, splicing factors localized throughout the nucleoplasm; when it was low, they concentrated in speckle-like domains. Thus, both splicing and transcription factors have a dynamic localization within the nucleus and may be recruited from speckles to the position of active transcription.

Our results are also in agreement with recent biochemical studies that suggest an interaction between the transcription and processing machinery. Isolation of cDNAs that interact with the CTD of polymerase II in the yeast two-hybrid system (Yuryev *et al.*, 1996) identified four genes each containing a significant arginine-serine domain similar to those found in the arginine-serine family of splicing factors (Zahler *et al.*, 1992), suggesting a direct protein-protein interaction between polymerase II and splicing factors. More directly, antibodies against polymerase II inhibit *in vitro* splicing and the pol II CTD

peptide inhibits *in vitro* (Yuryev *et al.*, 1996) and *in vivo* (Du and Warren, 1997) RNA splicing.

Models for nuclear organization of mRNA transcription and processing

This study examined the distribution of bulk factors. Earlier attempts to address the possibility of co-localization of transcription and processing have used *in situ* hybridization techniques to detect identified individual transcripts or bulk polyadenylated RNA. Although different laboratories have observed different results, at least several abundant, actively transcribed genes have consistently been localized to speckles rather than to the inter-speckle meshwork described in this report. Two possible explanations present themselves immediately for the discrepancy between localization of individual transcripts to speckles and the localization of bulk transcription to the region between the speckles. The first explanation, which we prefer, is that transcription units operating to produce extremely abundant pre-mRNAs are situated near localized regions of high concentration of splicing factors—the 'speckles'—but that most transcription units operating at lower efficiency are localized throughout the nucleoplasm. Certainly the genes that have been localized to speckles in previous studies, collagen and fibronectin, are extremely abundant RNAs in the cell types examined and might not be representative of lower copy transcription units (Xing *et al.*, 1993, 1995). The ability of high-activity transcription units to be found near speckles is underscored by the co-localization of speckles and transfected plasmid DNA with transcription driven from strong promoters (Huang and Spector, 1996).

A second, and in our opinion less attractive, hypothesis is that BrU incorporation and polymerase II localization cannot be used to indicate pre-mRNA synthesis because of the existence of a large population of polymerase II-driven transcription to produce RNAs other than pre-mRNAs. Early studies examining nuclear RNA indicated that the sequence complexity of nuclear RNA is not represented in mRNA (Harpold *et al.*, 1981; Salditt-Georgieff *et al.*, 1981). Given the experimental limitation used in early studies, however, this non-mRNA may in part be represented by intervening sequences (whose sizes are considerably larger than exons in vertebrates) as well as a class of RNA undergoing rapid nuclear turnover (from genes regulated at the level of nuclear RNA stabilization). In this study we document that incorporation of ribonucleotides and pol II-localization as detected by antibodies directed against a transcriptionally competent form of pol II are not restricted to speckles, but are instead widely distributed throughout the nucleoplasm, indicating that transcription by pol II is broadly distributed. Thus, in the absence of contradictory data establishing that pol II distribution and BrU incorporation represent a bulk population of RNA other than pre-mRNA, we interpret our results to indicate that transcription and processing occur throughout the nucleoplasm.

In addition to the broad meshwork pattern observed for most of the antibodies used in this study, we likewise observed that each also stained multiple sites of preferred localization. These were more numerous and smaller in diameter than classic speckles. For polymerase II, these 'dots' overlapped with BrU incorporation, indicating that they represent active sites of *in situ* transcription. A similar

pattern of preferred staining has been noticed previously in *in situ* incorporation experiments (Jackson *et al.*, 1993; Wansink *et al.*, 1993; Iborra *et al.*, 1996; Schul *et al.*, 1996). The identity of these regions is unclear, but they are of an abundance to suggest that they may represent abundant transcription units. The association of these domains with the polyadenylation factor CstF underscores their likely identification as transcription units. Splicing factor antibodies only stained a subset of these locations, suggesting preferred localization of splicing factors with only a subset of pre-mRNAs.

Overall, our results are not consistent with speckles as the preferred sites of transcription and RNA processing. Incorporating our results with those of other laboratories indicating an ability to locate individual transcription units to speckles, we suggest that transcription and splicing are coincident and localized throughout the nucleoplasm, presumably at the sites of the transcribed chromatin. In addition, we suggest that abundant transcription units, especially those driven from transfected genes, can be seen at the light microscopy level as dots or speckles of preferred staining that represent local high concentrations of both transcription and processing factors. Furthermore, we suggest a dynamic interaction between the dispersed population and the clustered population that reflects transcriptional status such that both transcriptional and processing factors can be found highly localized when transcription is minimal and highly dispersed when transcription is maximal. Combining these two suggestions indicates that speckles may be factor recycling centers which associate closely with some highly active transcription units because of the abundance of factors within the speckles.

Materials and methods

Cell culture and heat shock

Human cervical epidermoid carcinoma cells (CaSki) were grown in RPMI 1640 with 10% fetal bovine serum (FBS). HeLa and MDCK cells were grown in DMEM with 10% FBS. The human ovary cancer cell line, OV-MZ-15 was maintained in the same DMEM/FBS medium supplemented with 10 mM HEPES pH 7.0, 2 mM glutamine, 116 µg/ml arginine and 30 µg/ml asparagine. Heat shock was applied by incubating MDCK cells grown on coverslips at 45°C for 1 h, followed by recovery for 1 h at 37°C or 0°C as a control.

Cross-linking of radiolabeled RNA to transcriptionally active Pol II in isolated nuclei and immunoprecipitation of labeled RNA with pol II antibodies

This procedure was adapted from an established protocol as described (Bartholomew *et al.*, 1986). Briefly, HeLa cell nuclei (5×10^7 /ml) were incubated in 'run on buffer' containing 500 mCi/ml [α - 32 P]CTP (>2000 Ci/mmol, Amersham), 0.1 mM ATP, 0.1 mM 4-thio-UTP, 1 mM-3'-O-methyl GTP, 0.12 M KCl, 5 mM Mg-acetate, 0.05 mM EDTA, 12.5% glycerol, 0.05 mM dithiothreitol, 25 mM Tris-HCl pH 8 and 0.1 mM PMSF. The transcription reaction was carried out at 20°C for 10 min with or without α -amanitin (10 µg/ml), after which the nuclei were exposed to UV ($\lambda_{\text{max}} = 310$ nm) to induce covalent cross-linking between proteins in contact with 4-thio-UTP-containing radiolabeled RNA. The nuclei were subsequently digested with RNase T1 and DNase I as described (Bartholomew *et al.*, 1986). A nuclear extract was prepared from these nuclei using ice-cold T-buffer (50 mM Tris-HCl pH 7.4, 250 mM NaCl, 1% Triton X-100, 1 mM PMSF, 0.2 mM Na_2VO_4 , 5 mM β -glycerolphosphate). Insoluble material was pelleted by centrifugation (15 000 g for 10 min), and the supernatant was used for immunoprecipitation with mAb H14 or H5 as described (Kim *et al.*, 1997). Immunoprecipitates were washed three times in ice-cold T-buffer, boiled in SDS sample buffer, subjected to 6% SDS-PAGE, and processed for autoradiography.

In situ run-on transcription

CaSki or OV-MZ-15 cells grown on coverslips were washed with a 'physiological' buffer (PB) (pH 7.4, 22 mM Na^+ , 130 mM K^+ , 1 mM Mg^{2+} , 2.5 mM Ca^{2+} , 31 mM Cl^- , 100 mM acetate, 11 mM phosphate, 0.5 mM ATP, 1 mM DTT and 0.2 mM PMSF; Jackson *et al.*, 1993). Samples were then slightly permeabilized with cold 0.025–0.05% Triton X-100 in PB for 2 min. Run-on transcription was performed by incubating cells with reaction buffer (PB containing 0.1 mM CTP, GTP and BrUTP; 2 mM MgCl_2 and 4 U/ml RNasin) for 20–30 min at 30°C. Alternatively, cells were incubated with reaction buffers containing mixtures of UTP/BrUTP in a ratio of 1/3, 2/2 or 3/1, or UTP alone, respectively. For inhibition of *in situ* transcription, 1–2 µg/ml α -amanitin was added to the reaction buffer during BrU incorporation. Reactions were stopped by rinsing samples with cold PB and cells were post-permeabilized with cold 0.1–0.2% Triton X-100 in PB for 2 min. Finally, cells were fixed in 3.2% paraformaldehyde for 20 min. The nascent RNA was detected by immunofluorescence with a commercial BrdU antibody which also recognizes BrU.

Immunofluorescence and microscopy

The following primary antibodies were used for immunofluorescence in this study: pol II large subunit antibodies, mAb H5 and mAb H14 (Bregman *et al.*, 1995); mAb anti-SC35 (ATCC); anti-BrdU mAbs (Boehringer Mannheim, Seralab); mAb anti CstF 64 kDa (Takagaki *et al.*, 1990); and mAb 104 (Zahler *et al.*, 1992).

Cells were labeled with BrUTP and then fixed as described above. For samples without BrU labeling, cells were permeabilized for 1–2 min with 0.5% Triton X-100 in CSK buffer (10 mM PIPES pH 7, 1 mM EGTA, 3 mM MgCl_2 , 20% sucrose; Fey *et al.*, 1986), and then fixed with 3.2% paraformaldehyde in the same buffer for 20 min. Alternatively, cells (Figure 6) were fixed and then permeabilized with 0.5% Triton X-100 in PBS. For fluorescent staining, coverslips were incubated with properly diluted antibodies at 37°C for 30 min. After washing with PBS three times, cells were incubated with diluted secondary antibodies (1:20–1:50) conjugated with FITC, TRITC or Texas Red (Jackson Immuno-Research) for 30 min, and then washed and counterstained with DAPI solution (0.1 µg/ml) for 5 min. For double staining, fluorescent antibodies were all pre-tested not to cross-react with other immunoglobulins. Samples were finally mounted with glycerol/PBS (9:1) containing 0.1% *p*-phenylenediamine (Sigma).

Images were obtained from fluorescent microscopy (Zeiss Axiophot; Figures 1B and C, and 3–5) or laser confocal microscopy (Molecular Dynamics; Figures 2 and 6). Optic sections of double-stained samples were scanned with a Nikon laser microscope. A dual wavelength channel was used to excite FITC and TRITC/Texas Red at 488 and 568 nm respectively. Fluorescent signals of both fluorochromes were recorded simultaneously at one scan. No cross-talk was observed. Image slides were taken from a digital camera (Polaroid, CI 5000S) using Adobe and Powerpoint software.

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References

- Bartholomew, B., Dahmus, M.E. and Meares, C.F. (1986) RNA contacts subunits I₁₀ and I₁₂ in HeLa RNA polymerase II transcription complexes. *J. Biol. Chem.*, **261**, 14226–14231.
- Beyer, A.L., Yvonne, N. and Osheim, Y.N. (1988) Splice site selection, rate of splicing, and alternative splicing on nascent transcripts. *Genes Dev.*, **2**, 754–765.
- Bregman, D.B., Du, L., van der Zee, S. and Warren, S.L. (1995) Transcription-dependent redistribution of the large subunit of polymerase II to discrete nuclear domains. *J. Cell Biol.*, **129**, 287–298.
- Carmo-Fonseca, M., Tollervey, D., Pepperkok, R., Barabino, S.M.L., Merdes, A., Brunner, C., Zamore, P.D., Breen, M.R., Hurt, E. and Lamond, A.I. (1991a) Mammalian nuclei contain foci which are highly enriched in components of the pre-mRNA splicing machinery. *EMBO J.*, **10**, 195–206.

- Carmo-Fonseca,M., Pepperkok,R., Sproat,B.S. Ansorge,W., Swanson,M.S. and Lamond,A.I. (1991b) *In vivo* detection of snRNP-rich organelles in the nuclei of mammalian cells. *EMBO J.*, **10**, 1863–1873.
- Carmo-Fonseca,M., Pepperkok,R., Carvalho,M.T. and Lamond,A.I. (1992) Transcription-dependent colocalization of the U1, U2, U4/U6, and U5 snRNPs in coiled bodies. *J. Cell Biol.*, **117**, 1–14.
- Carter,K.C., Taneja,K.L. and Lawrence,J.B. (1991) Discrete nuclear domains of poly (A) RNA and their relationship to the functional organization of the nucleus. *J. Cell Biol.*, **115**, 1191–1202.
- Carter,K.C., Bowman,D., Carrington,W., Fogarty,K., McNeil,J.A., Fay,F.S. and Lawrence,J.B. (1993) A three-dimensional view of precursor messenger RNA metabolism within the mammalian nucleus. *Science*, **259**, 1330–1335.
- Corden,J.L. (1993) RNA polymerase transcription cycles. *Curr. Opin. Gen. Dev.*, **3**, 213–218.
- Dahmus,M.E. (1995) Phosphorylation of the C-terminal domain of RNA polymerase II. *Biochim. Biophys. Acta*, **1261**, 171–182.
- Du,L. and Warren,S.L. (1997) A functional interaction between the carboxy terminal domain of RNA polymerase II and pre-mRNA splicing. *J. Cell Biol.*, in press.
- Fakan,S. (1994) Perichromatin fibrils are *in situ* forms of nascent transcripts. *Trends Cell Biol.*, **4**, 86–90.
- Fakan,S. and Puvion,E. (1980) The ultrastructural visualization of nuclear and extranuclear RNA synthesis and distribution. *Int. Rev. Cytol.*, **65**, 255–299.
- Fey,E.G., Krochmalnic,G. and Penman,S. (1986) The nonchromatin substructures of the nucleus the ribonucleoprotein (RNP)-containing and RNP-depleted matrices analyzed by sequential fractionation and resinless section microscopy. *J. Cell Biol.*, **102**, 1654–1665.
- Fu,X.-D. and Maniatis,T. (1990) Factor required for mammalian spliceosome assembly is localized to discrete regions in the nucleus. *Nature*, **343**, 437–444.
- Harpold,M.M., Wilson,M.C. and Darnell,J.E., Jr (1981) Chinese hamster polyadenylated messenger ribonucleic acid: relationship to non-polyadenylated sequences and relative conservation during messenger ribonucleic acid processing. *Mol. Cell Biol.*, **1**, 188–198.
- Hendzel,M.J. and Bazett-Jones,D.P. (1995) RNA polymerase II transcription and the functional organization of the mammalian cell nucleus. *Chromosoma*, **103**, 509–516.
- Huang,S. and Spector,D. (1991) Nascent pre-mRNA transcripts are associated with nuclear regions enriched in splicing factors. *Genes Dev.*, **5**, 2288–2302.
- Huang,S. and Spector,D. (1992) U1 and U2 small nuclear RNAs are present in nuclear speckles. *Proc. Natl Acad. Sci. USA*, **89**, 305–308.
- Huang,S. and Spector,D.L. (1996) Intron-dependent recruitment of pre-mRNA splicing factors to sites of transcription. *J. Cell Biol.*, **144**, 719–732.
- Iborra,F.J., Pombo,A., Jackson,D.A. and Cook,P.R. (1996) Active RNA polymerases are localized within discrete transcription ‘factories’ in human nuclei. *J. Cell Sci.*, **109**, 1427–1436.
- Jackson,D.A., Hassan,A.B., Errington,R.J. and Cook,P.R. (1993) Visualization of focal sites of transcription within human nuclei. *EMBO J.*, **12**, 1059–1065.
- Jenny,A., Jauri,J.P. and Keller,W. (1994) Characterization of cleavage and polyadenylation specificity factor and cloning of its 100-kilodalton subunit. *Mol. Cell Biol.*, **14**, 8183–8190.
- Kang,M.E. and Dahmus,M.E. (1993) RNA polymerase IIA and IIO have distinct roles during transcription from TATA-less murine dihydrofolate reductase promoter. *J. Biol. Chem.*, **268**, 25033–25040.
- Kim,E., Lei,D., Bregman,D.B. and Warren,S.L. (1997) Splicing factors associate with hyperphosphorylated RNA polymerase II in the absence of pre-mRNA. *J. Cell Biol.*, in press.
- Krause,S., Fakan,S., Weis,K. and Wahle,E. (1994) Immunodetection of poly(A) binding protein II in the cell nucleus. *Exp. Cell Res.*, **214**, 75–82.
- Lawrence,J.B., Carter,K.C. and Xing,Y. (1993) Probing functional organization within the nucleus, is genome structure integrated with RNA metabolism? *Cold Spring Harbor Symp. Quant. Biol.*, **58**, 807–818.
- Laybourn,P.J. and Dahmus,M.E. (1990) Phosphorylation of RNA polymerase IIA occurs subsequent to interaction with the promoter and before the initiation of transcription. *J. Biol. Chem.*, **265**, 13165–13173.
- Möbus,V.J., Gerharz,C.D., Press,U., Moll,R., Beck,T., Mellin,W., Pollow,K., Knapstein,P.G. and Kreienberg,R. (1994) Morphological, immunohistochemical and biochemical characterization of 6 newly established human ovarian cancer cell lines. *Int. J. Cancer*, **52**, 76–84.
- Moen,P.T., Jr, Smith,K.P. and Lawrence,J.B. (1995) Compartmentalization of specific pre-mRNA metabolism: an emerging view. *Hum. Mol. Genet.*, **4**, 1779–1789.
- Nyman,U., Hallman,H., Hadlaczy,G., Pettersson,I., Sharp,G. and Ringertz,N.R. (1986) Intracellular localization of snRNP antigens. *J. Cell Biol.*, **102**, 137–144.
- Salditt-Georgieff,M., Harpold,M.M., Wilson,M.C. and Darnell,J.E., Jr (1981) Large heterogeneous nuclear ribonucleic acid has three times as many 5' caps as polyadenylic acid segments, and most caps do not enter polyribosomes. *Mol. Cell Biol.*, **1**, 179–187.
- Schul,W., Groenhout,B., Koberna,K., Takagaki,Y., Jenny,A., Manders,E.M.M., Raska,I., van Driel,R. and de Jong,L. (1996) The RNA 3' cleavage factors CstF 64 kDa and CPSF 100 kDa are concentrated in nuclear domains closely associated with coiled bodies and newly synthesized RNA. *EMBO J.*, **15**, 2883–2892.
- Spector,D.L. (1984) Colocalization of U1 and U2 small nuclear RNPs by immunocytochemistry. *Biol. Cell*, **51**, 109–112.
- Spector,D.L. (1990) Higher order nuclear organization, three-dimensional distribution of small nuclear ribonucleoprotein particles. *Proc. Natl Acad. Sci. USA*, **87**, 147–152.
- Spector,D.L. (1993) Macromolecular domains within the cell nucleus. *Annu. Rev. Cell Biol.*, **9**, 265–315.
- Spector,D.L., Fu,X.-D. and Maniatis,T. (1991) Associations between distinct pre-mRNA splicing components and the cell nucleus. *EMBO J.*, **10**, 3467–3481.
- Takagaki,Y., Manley,J.L., MacDonald,C.C., Wilusz,J. and Shenk,T. (1990) A multisubunit factor, CstF, is required for polyadenylation of mammalian pre-mRNAs. *Genes Dev.*, **4**, 2112–2120.
- van Driel,R., Wansink,D.G., van Steensel,B., Grande,M.A., Schul,W. and de Jong,L. (1995) Nuclear domains and the nuclear matrix. *Int. Rev. Cytol.*, **162A**, 151–189.
- Wang,J., Cao,L.G., Wang,Y.L. and Pederson,T. (1991) Localization of pre-messenger-RNA at discrete nuclear sites. *Proc. Natl Acad. Sci. USA*, **88**, 7391–7395.
- Wansink,D.G., Schul,W., der Kraan,I., van Steensel,B., van Driel,R. and de Jong,L. (1993) Fluorescent labeling of nascent RNA reveals transcription by RNA polymerase II in domains scattered throughout the nucleus. *J. Cell Biol.*, **122**, 283–293.
- Wansink,D.G., Nelissen,R.L.H. and de Jong,L. (1994) *In vitro* splicing of pre-mRNA containing bromouridine. *Mol. Biol. Rep.*, **19**, 109–113.
- Weeks,J.R., Jardin,S.E., Shen,J., Lee,J.M. and Greenleaf,A.L. (1993) Locus-specific variation in phosphorylation state of RNA polymerase II *in vivo*: correlations with gene activity and transcript processing. *Genes Dev.*, **7**, 2329–2344.
- Xing,Y., Johnson,C.V., Dobner,P. and Lawrence,J.B. (1993) Higher level organization of individual gene transcription and RNA splicing, integration of nuclear structure and function. *Science*, **259**, 1326–1330.
- Xing,Y., Johnson,C.V., Moen,P.T., McNeil,J.A. and Lawrence,J.B. (1995) Nonrandom gene organization, structural arrangements of specific pre-mRNA transcription and splicing with the SC-35 domains. *J. Cell Biol.*, **131**, 1635–1647.
- Yuryev,A., Patturajan,M., Litingtung,Y., Joshi,R.V., Gentile,C., Gebara,M. and Corden,J.L. (1996) The C-terminal domain of the largest subunit of RNA polymerase II interacts with a novel set of serine/arginine-rich proteins. *Proc. Natl Acad. Sci. USA*, **93**, 6975–6980.
- Zahler,A.M., Lane,W.S., Stolk,J.A. and Roth,M.B. (1992) S/R proteins – a conserved family of pre-messenger-RNA splicing factors. *Genes Dev.*, **6**, 837–847.
- Zhang,G., Taneja,K.L., Singer,R.H. and Green,M.R. (1994) Localization of pre-mRNA splicing in mammalian nuclei. *Nature*, **372**, 809–812.

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