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RCC1 and Nuclear Organization

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We have examined the effect of RCC1 function on the nuclear organization of pre-mRNA splicing factors and poly(A)$^+$ RNA in the tsBN2 cells, a RCC1 temperature-sensitive mutant cell line. We have found that at 4–6 h after shifting cells from the permissive temperature (32.5°C) to the restrictive temperature (39.5°C), both small nuclear ribonucleoprotein particles and a general splicing factor SC35 reorganized into 4–10 large round clusters in the nucleus, as compared with the typical speckled distribution seen in cells at the permissive temperature. In situ hybridization to poly(A)$^+$ RNA resulted in a similar pattern. Examination by double labeling demonstrated that the redistribution of splicing factors coincides with that of poly(A)$^+$ RNA. Such changes in the nuclear organization of splicing factors and poly(A)$^+$ RNA were not the result of the temperature shift or of chromatin condensation. Cellular transcription was not significantly altered in these cells and extracts made from both the permissive and restrictive temperature were splicing competent. Electron microscopic examination demonstrated that the large clusters containing both splicing factors and poly(A)$^+$ RNA were fused interchromatin granule clusters. In addition, small electron-dense dot-like structures measuring approximately 80 nm in diameter were also observed, most of which are accumulated in enlarged interchromatin granule clusters in the nucleoplasm of RCC1$^-$ cells. In spite of the significant changes observed in the nucleoplasm, relatively little alteration was observed in nucleolar structure by both light and electron microscopic examination. The above observations suggest that the RCC1 protein directly or indirectly regulates the organization of splicing components and poly(A)$^+$ RNA in the cell nucleus and that RCC1 may play a role in nuclear organization.

INTRODUCTION

TsBN2 cells, originally isolated from BHK21/13 hamster cells, have a temperature-sensitive mutation in the RCC1 (regulator of chromosomal condensation) gene. Initial observations revealed a premature chromosome condensation phenotype (PCC) when these cells were grown at the restrictive temperature (Nishimoto *et al.*, 1978; Uchida *et al.*, 1990; Nishitani *et al.*, 1991). Subsequent studies have shown that the loss of RCC1 function at different points in the cell cycle gives rise to different phenotypes. When cells are shifted to the restrictive temperature in the S-phase, the RCC1 mutation results in an interruption of DNA replication, PCC, and a premature mitosis (Nishimoto *et al.*, 1978; Nishitani *et al.*, 1991). When cells are shifted to the restrictive temperature in G_1, they are unable to pass the G_1-S-phase boundary (Nishimoto *et al.*, 1978). During the S-phase, the loss of RCC1 function induces the dephosphorylation of $p34^{cdc2}$ complexed with cyclin B and the activation of its histone H1 kinase activity. These changes do not occur in G_1 cells when grown at the restrictive temperature (Pines and Hunter, 1989; Moreno *et al.*, 1990; Nishitani *et al.*, 1991). The pleiotropic phenotype resulting from the RCC1 mutation suggests that the RCC1 protein affects multiple downstream regulatory pathways. *RCC1* genes and cDNAs have been cloned in human and hamster cells and their homologues in *Drosophila*, *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe* have also been isolated and characterized (for a review, see Dasso, 1993). The *RCC1* gene encodes an abundant nuclear DNA-binding protein that interacts with a small GTP-bind-

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ing protein, ran or TC4 in mammalian cells, spil in *S. pombe*, and GSP1 and GSP2 in *S. cerevisiae* (Bischoff and Ponstingl, 1991; Matsumoto and Beach, 1991; Coutavas *et al.*, 1993; Saitoh and Dasso, 1995; Azuma *et al.*, 1996). Both proteins are required for normal cell cycle regulation (Matsumoto and Beach, 1991; Sazer and Nurse, 1994).

In addition to being essential for DNA replication, the initiation of mitosis, and the transition from mitosis to G_1, the RCC1/ran proteins have also been shown to be involved in protein import into the nucleus (Melchior *et al.*, 1993; Moore and Blobel, 1993; Tachibana *et al.*, 1994; Schlenstedt *et al.*, 1995; Yokoyama *et al.*, 1995; Gorlich *et al.*, 1996; Saitoh *et al.*, 1996), RNA transcription, processing, and transport (for a review, see Dasso, 1993). In budding yeast, a mutation in prp20 (an RCC1 homologue) causes an accumulation of longer mRNA species (Aebi *et al.*, 1990) and affects splicing of actin pre-mRNA. In addition, Forrester *et al.* (1992) have found that prp20 mutants are defective in 3′-end cleavage of several different mRNAs and in processing RNAs at their normal polyadenylation sites. prp20 mutants also exhibit an abnormal transcription initiation and an inhibition of poly(A)$^+$ RNA transport from the nucleus to the cytoplasm (Forrester *et al.*, 1992; Amberg *et al.*, 1993; Kakowaki *et al.*, 1993). In tsBN2 cells, the bulk RNA transcription rate is not significantly reduced within 12 h of inactivation of the RCC1 protein (Nishimoto *et al.*, 1978). However, the transport of poly(A)$^+$ RNA is blocked when cells are grown at the restrictive temperature (Amberg *et al.*, 1993; Kakowaki *et al.*, 1993; Schlenstedt *et al.*, 1995). More recently, GTP-Ran has been shown to be essential for the nucleocytoplasmic transport of precursors of small nuclear RNAs (snRNAs), intranuclear transport of U3 snRNA, and processing of ribosomal RNAs (Cheng *et al.*, 1995). These studies have demonstrated that RCC1/ran may play diverse and important roles in nuclear RNA metabolism.

Because RCC1/ran function clearly affects nuclear pre- and mRNA metabolism, we were interested in determining whether loss of RCC1 function would affect the nuclear organization of pre-mRNA splicing factors and poly(A)$^+$ RNA, as well as nuclear structure in general. Pre-mRNA splicing has been extensively characterized at the biochemical level in vivo and in vitro (for reviews, see Moore *et al.*, 1993; Kramer, 1996). At the cellular level, the organization of small nuclear ribonucleoprotein particles (snRNPs) and non-snRNP splicing factors has been examined by immunocytochemistry and in situ hybridization. Localization studies using antibodies that specifically recognize snRNP antigens and non-snRNP splicing factors, such as SC35 and SF2/ASF, have shown that these splicing factors are distributed in a speckled pattern in the interphase nucleus and that snRNPs are also present in coiled bodies (for a review, see Spector, 1993). In situ hybridization with oligonucleotides complementary to the major spliceosomal snRNAs has demonstrated a similar localization pattern (Carmo-Fonseca *et al.*, 1991, 1992; Huang and Spector, 1992).

The speckled distribution of splicing factors corresponds to both interchromatin granule clusters and perichromatin fibrils at the electron microscopic level (Fakan *et al.*, 1984; Spector *et al.*, 1991). Three-dimensional reconstruction of whole nuclei has shown that some of the interchromatin granule clusters and perichromatin fibrils interconnect (Spector, 1990; Spector *et al.*, 1991). Interchromatin granule clusters do not appear to be labeled after short pulses of [^{3}H]uridine incorporation (Fakan and Bernhard, 1971; Fakan and Nobis, 1978) and exhibit little to no labeling with anti-DNA antibodies (Turner and Franchi, 1987), suggesting that these clusters are not the sites of active RNA transcription. Instead, they may represent storage and/or reassembly sites for snRNPs and non-snRNP splicing factors (Jiménez-García and Spector, 1993). Perichromatin fibrils, however, are labeled with [^{3}H]uridine (for reviews, see Fakan and Puvion, 1980; Fakan, 1994), suggesting that they represent nascent RNA transcripts. The elaborate organization of splicing factors in a speckled pattern appears to be functionally related to the transcriptional and splicing activities of the cell (for a review, see Huang and Spector, 1996a). When cells are infected with adenovirus 2 or transiently transfected with expression vectors transcribing intron-containing RNAs, splicing factors are recruited to the newly introduced and highly active transcription sites (Jiménez-García and Spector, 1993; Huang and Spector, 1996b). In addition, splicing factors are reorganized into large interchromatin granule clusters upon inhibition of transcription or splicing (Spector *et al.*, 1993; O'Keefe *et al.*, 1994). These observations suggested that the nuclear organization of splicing factors is dynamic and that these factors shuttle between storage and/or reassembly sites (interchromatin granule clusters) and sites of active transcription and pre-mRNA splicing (perichromatin fibrils) (Jiménez-García and Spector, 1993; O'Keefe *et al.*, 1994; Huang and Spector, 1996b). In this article, we report that a cellular factor, the RCC1 protein, is important for the maintenance of the speckled distribution of splicing factors and poly(A)$^+$ RNA. The loss of RCC1 function induces a significant change in the nuclear distribution of these components and changes in nuclear structure in general at the ultrastructural level.

MATERIALS AND METHODS

Cell Culture and Preparation

TsBN2 and BHK (baby hamster kidney) cells were grown in DMEM supplemented with 10% fetal bovine serum (Life Technologies, Gaithersburg, MD) and maintained at 32.5°C with 7.5% CO_2. Cells were grown on glass coverslips in 35-mm-diameter Petri dishes for immunolabeling and in situ hybridization experiments. When cells reached subconfluence, some of the tsBN2 and BHK cells were shifted to the restrictive temperature, 39.5°C, for various lengths of time (i.e., 4, 6, 8, 10, and 12 h). Cells were synchronized to the G_0-G_1

boundary by growth in serum-free medium for 36 h. Cells were then released into G_1 by replacing this medium with complete medium containing 10% fetal bovine serum. Upon entry into G_1, cells were shifted to the restrictive temperature.

In Situ Hybridization

Cells incubated at both the permissive and the restrictive temperatures were prepared for fluorescence in situ hybridization as described previously (Huang *et al.*, 1994). Briefly, cells were rinsed with phosphate-buffered saline (PBS) and fixed in 4% formaldehyde for 15 min at room temperature. Cells were then washed for three 15-min periods in PBS and permeabilized with 0.5% Triton X-100 for 5 min at 4°C. Cells were again rinsed with two 15-min periods in PBS. An oligo(dT) 50-mer (dT_{50}) 3′ end labeled with fluorescein isothiocyanate (FITC; Operon Technol, Alameda, CA) was used as a probe for in situ hybridization to poly(A)$^+$ RNA. Cells were equilibrated in 2× standard saline citrate (SSC) for 10 min and were then subjected to hybridization. The hybridization mixture contained 80 ng/20 μl dT_{50}, 2× SSC, 1 mg/ml tRNA, 10% dextran sulfate, and 25% formamide. Hybridization was performed at 42°C in a humidified chamber overnight. After hybridization, cells were washed for two 15-min periods in 2× SSC and for one 15-min period in 0.5× SSC.

Immunolabeling

After hybridization, cells were washed for three 10-min periods in PBS and incubated with anti-SC35 primary antibody ascites (Fu and Maniatis, 1990) at a dilution of 1:1000 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.

Cells probed with antibodies alone were fixed, permeabilized as described above, and then incubated with monoclonal antibody specific to snRNPs (Y12 at a 1:1000 dilution) and a human antibody specific to nucleoli (fibrillarin at a 1:5 dilution; Ochs *et al.*, 1985) for 1 h at room temperature. After subsequent washing in PBS, cells were incubated with the corresponding FITC- or Texas Red-conjugated secondary antibody for 1 h at room temperature. Cells were then washed for three 10-min periods with PBS.

Coverslips were mounted onto glass slides with mounting medium containing 90% glycerol in 0.2 M Tris base (pH 8.0) with 1 mg/ml paraphenylenediamine as an antifading agent. Cells were examined with a Nikon FXA microscope equipped with epifluorescence and differential interference contrast (DIC) optics.

In Situ Autoradiography

Cells were incubated with [^{3}H]uridine (50 μCi/ml; Amersham) in culture medium for 10 min at the corresponding temperature followed by washing with medium containing 1 mg/ml unlabeled uridine for two 5-min periods. Cells were then fixed in 2% formaldehyde in PBS (pH 7.3) and washed with PBS. A monolayer of Ilford K5D emulsion was subsequently applied to each coverslip and allowed to dry for 1 h in the dark. Coverslips were stored at 4°C and developed after a 20-d exposure. They were developed for 7 min in a solution of 7.5 g of Metol, 5.0 g of anhydrous sodium sulfite, and 2.0 g of potassium thiocyanate per 1000 ml of distilled water. Coverslips were then washed in distilled water for 1 min and fixed for 5 min in Kodak fixer. After washing in several changes of distilled water for 30 min, coverslips were mounted and examined with a 60×/1.4 numerical aperature objective on a Nikon FXA microscope.

In Vitro Splicing Assays

Nuclear extracts for in vitro splicing were prepared as described by Dignam *et al.* (1983) from tsBN2 cells grown either at the permissive temperature or shifted to the restrictive temperature for 6 h. The plasmid containing the human β-globin gene, pSP64-HβΔ6 (Krainer *et al.*, 1984), was linearized with *Bam*HI and used as template DNA. ^{32}P-labeled 7-methyl-guanasine (^{7Me}GpppG) capped pre-mRNA substrate was prepared in runoff transcription with SP6 RNA polymerase as described (Krainer and Maniatis, 1985). In vitro splicing reactions in 25 μl with 1 or 5 μl of the nuclear extracts and 20 fmol of β-globin pre-mRNA were incubated at 30°C for 4 h as described previously (Mayeda and Ohshima, 1988). RNA products were analyzed by electrophoresis on a 5.5% polyacrylamide/7 M urea gel followed by autoradiography with an intensifying screen at −70°C as described (Mayeda and Ohshima, 1988).

Electron Microscopy

For examination by electron microscopy, tsBN2 cells grown at the permissive temperature and cells grown for 4 h at the restrictive temperature were rinsed with PBS and fixed in 4% formaldehyde/0.5–2% glutaraldehyde in PBS. Cells were then washed with PBS and dehydrated with increasing concentrations of ethanol. Cells were infiltrated and embedded in Epon-Araldite or LR White overnight at 60°C. Embedded cells were thin sectioned, poststained with 2% uranyl acetate and lead citrate, and examined with a Hitachi H-7000 transmission electron microscope operated at 75 kV.

To detect the non-snRNP splicing factor, SC35, by immunogold labeling, thin LR White sections were incubated with the monoclonal antibody at a 1:30 dilution overnight in Tris-buffered saline (0.02 M Tris, 0.15 M sodium chloride, 0.02 M sodium azide, 1% Tween 20), pH 7.6, at 4°C with 5% normal goat serum followed by incubation with 15 nm gold-conjugated goat anti-mouse antibodies at a dilution of 1:20 for 1 h at room temperature. Sections were then washed, poststained as above, and examined with a Hitachi H-7000 transmission electron microscope operated at 75 kV.

RESULTS

snRNP and Non-snRNP Splicing Factors Are Reorganized in tsBN2 Cells at the Restrictive Temperature When RCC1 Function Is Lost

Previous studies have shown that loss of RCC1 function results in the formation of irregular RNA transcripts in budding yeast and an impairment of RNA transport in budding and fission yeast and in mammalian cells (Forrester *et al.*, 1992; Amberg *et al.*, 1993; Kakowaki *et al.*, 1993; Cheng *et al.*, 1995; Schlenstedt *et al.*, 1995). We were interested in examining the nuclear organization of splicing factors in tsBN2 cells when RCC1 function is lost. Cells synchronized at the G_0-G_1 boundary by serum starvation or cells in a random population were shifted to the restrictive temperature (39.5°C) from the permissive temperature (33.5°C) for 4, 6, 8, 10, or 12 h. Cells were then processed for indirect immunofluorescence detection of the localization of snRNPs and the non-snRNP splicing factor SC35. In predominantly G_1 cells, a change in the nuclear organization of splicing factors was observed in cell nuclei after 4 h of incubation at the restrictive temperature. Both the non-snRNP splicing factor SC35 (Figure 1E) and the major snRNPs (Figure 1G) were reorganized into large clusters in some cells, as compared with the typical speckled pattern (Figure 1, A and C) observed in cells grown at the permissive temperature. Within 6 h, the majority of cells displayed a similar phenotype. The reorganization of

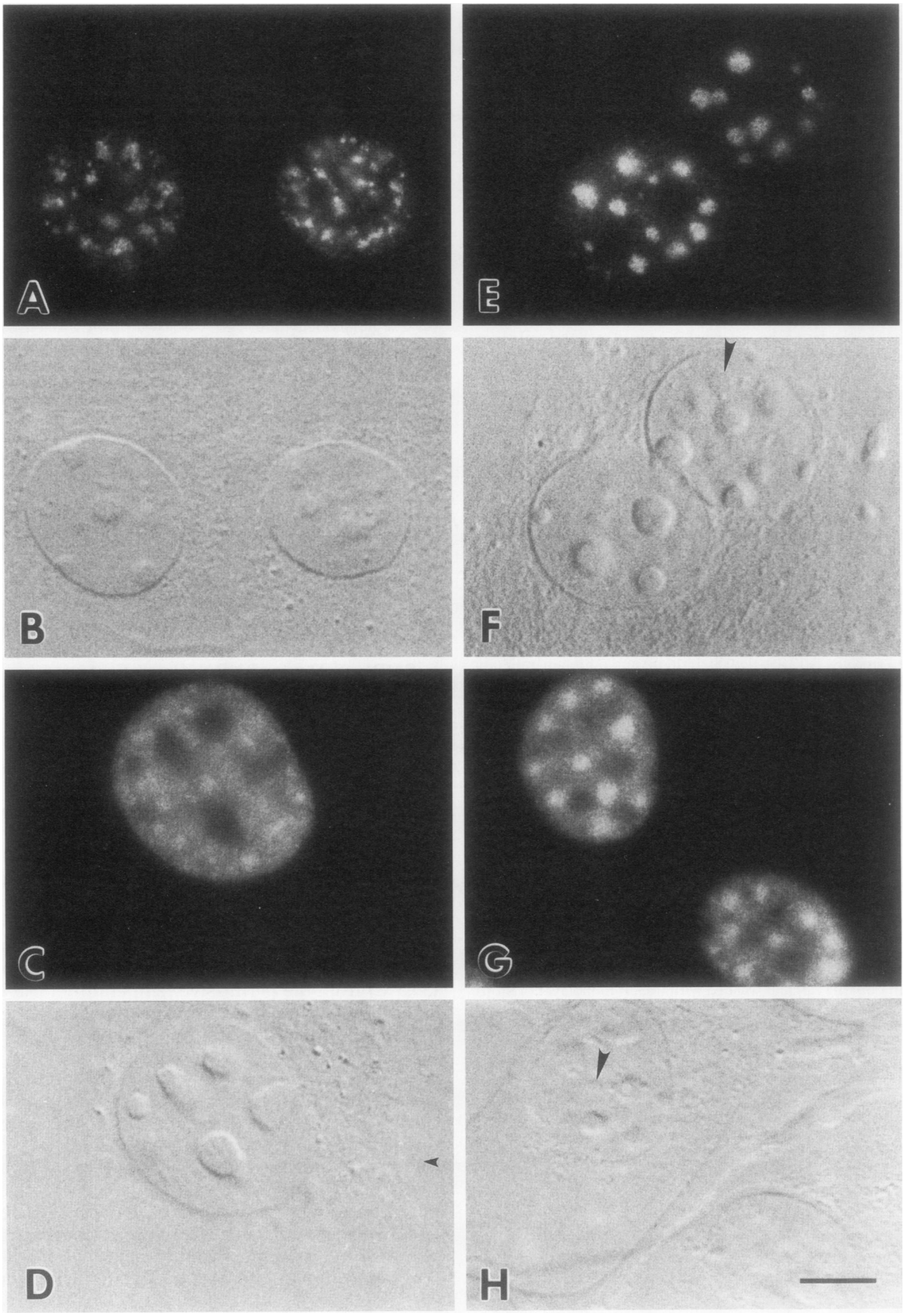

Figure 1.

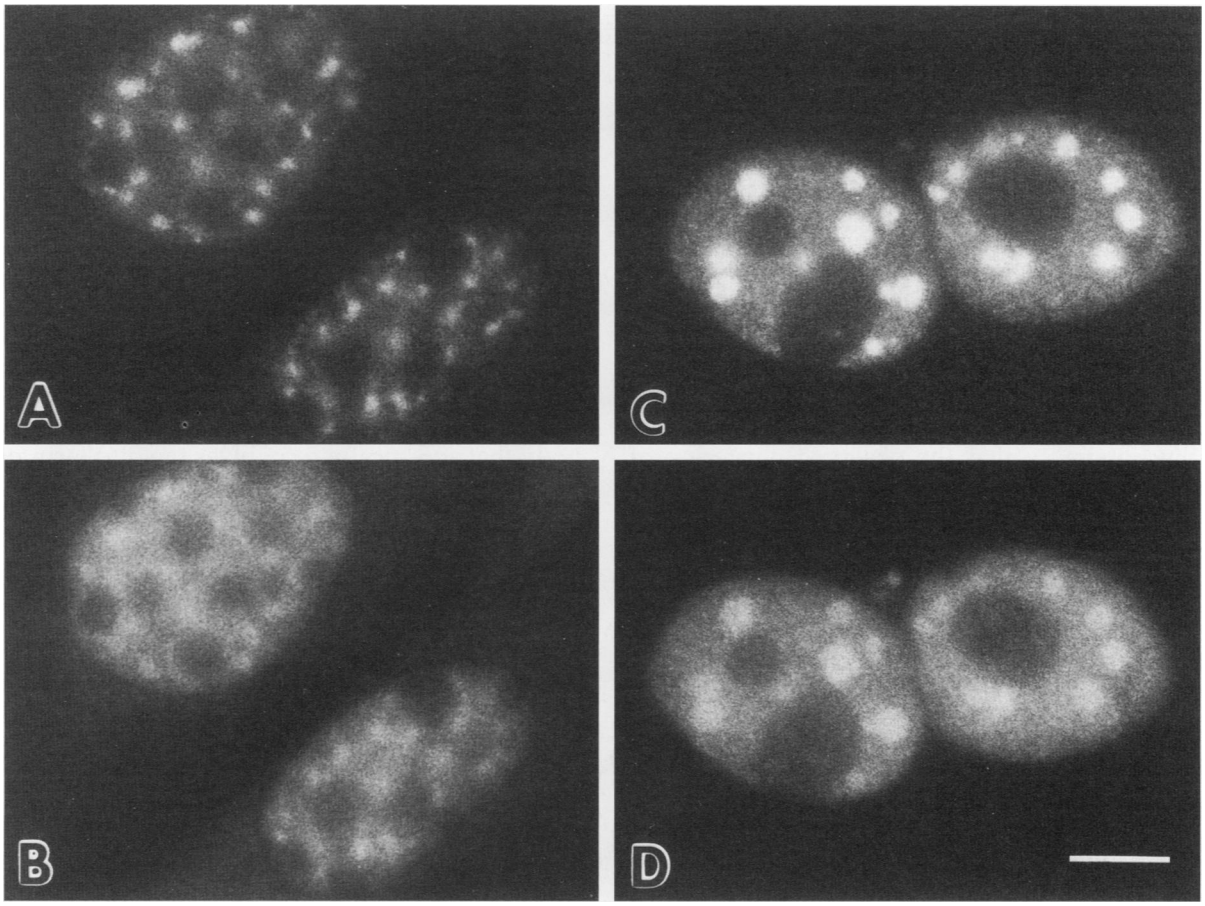

Figure 2. Distribution of poly(A)$^+$ RNA is changed in a manner similar to that of the organization of splicing factors in response to the loss of RCC1 function. The localization of SC35 (A and C) and poly(A)$^+$ RNA (B and D) were detected simultaneously in cells grown at both the permissive (A and B) and the restrictive temperature (C and D). Bar, 10 μm.

splicing factors into large clusters was so significant that visible bumps were observed in DIC images (Figure 1, F and H, arrowheads). In addition to large clusters, a diffuse staining of snRNPs was also observed in the nucleoplasm.

The Distribution of Poly(A)$^+$ RNA Is also Altered in Cells Grown at the Restrictive Temperature

Previous findings have shown that pre-mRNA transcription and splicing are closely linked (Beyer *et al.*, 1980; Beyer and Osheim, 1988; Huang and Spector, 1991; Xing *et al.*, 1993; Xing and Lawrence, 1993; O'Keefe *et al.*, 1994; McCracken *et al.*, 1997). Therefore, the dramatic alteration in splicing factor organization in RCC1$^-$ cells may also reflect or affect the metabolism of newly synthesized or preexisting RNA in the nucleus. To investigate the distribution of poly(A)$^+$ RNA in the absence of RCC1 function, we used a dT_{50} probe end-labeled with FITC to detect poly(A)$^+$ RNA in tsBN2 cells at both the permissive and the restrictive temperatures. The specificity of in situ hybridization using a dT_{50} probe has been previously evaluated (Huang *et al.*, 1994). When cells were released into G_1 and grown at the restrictive temperature for 4 h or longer, the distribution of poly(A)$^+$ RNA was changed into fewer and larger clusters in the cell nucleus in addition to being diffusely distributed in the nucleoplasm (Figure 2D). This change appeared to be similar to the change observed in the organization of splicing factors (Figure 1). Double labeling with a monoclonal antibody specifically recognizing SC35 showed that

Figure 1 (facing page). snRNP and non-snRNP splicing factors are reorganized in the tsBN2 cell nucleus upon RCC1 inactivation. When cells were grown at the permissive temperature, 33.5°C (A–D), both SC35 (A) and snRNPs (C) were localized in a speckled pattern in the nucleus. When G_1 cells were shifted to the restrictive temperature, 39.5°C (E–H) for 4 h, SC35 (E) and snRNPs (G) were reorganized into larger and fewer clusters. In the corresponding DIC images (F and H), visible bumps (arrowheads) can be observed in the nuclei that correspond to the sites where the splicing factors clustered. Bar, 10 μm.

SC35 (Figure 2, A and C) and poly(A)$^+$ RNA (Figure 2, B and D) are colocalized in cells at both the permissive (Figure 2, A and B) and the restrictive temperatures (Figure 2, C and D). These observations demonstrated that both splicing factors and poly(A)$^+$ RNA were similarly reorganized into fewer and larger clusters in response to the loss of RCC1 function.

The Changes in the Distribution of Splicing Factors and Poly(A)$^+$ RNA Are Not the Result of DNA Condensation or Temperature Change

One of the phenotypes of RCC1$^-$ cells is PCC during S phase. Although the above observations were largely made in G_0-G_1 cells, it was necessary to exclude the possibility that the observed clustering of splicing factors and poly(A)$^+$ RNA resulted from nonspecific aggregations due to chromatin condensation. We therefore examined tsBN2 cells for the localization of splicing factors, poly(A)$^+$ RNA, and DNA simultaneously in a random population of cells grown at the permissive or the restrictive temperatures. Cells that were labeled for splicing factors (Figure 3, A and D), or for poly(A)$^+$ RNA (our unpublished observation) were counterstained with 4,6-diamidino-2-phenylindole (DAPI), which specifically labels double-stranded DNA (Figure 3, B and E). In cells where snRNPs were reorganized into larger clusters after incubation at the restrictive temperature (Figure 3D), the DAPI staining of the same cells (Figure 3E) did not show chromatin condensation, as compared with cells that were grown at the permissive temperature (Figure 3B). Interestingly, the clusters that were labeled intensely with anti-snRNP antibodies and with the oligo(dT) probe (Figure 3D) contained little to no DNA (Figure 3E). These results demonstrated that the rearrangement of splicing factors and poly(A)$^+$ RNA did not result from DNA condensation. In fact, in cells with PCC, condensed chromatin (Figure 3E, the cell on the left), splicing factors were found predominantly in the cytoplasm (Figure 3D, the cell on the left). Normally, splicing factors disperse into the whole cell only when the nuclear envelope breaks down during mitosis. In the cell where condensed chromatin significantly changed the gross morphology of the nucleus, splicing factors redistributed to the cytoplasm, suggesting that the redistribution was likely the result of chromatin condensation and/or leakiness of the nuclear envelope.

Furthermore, to exclude the possibility that the reorganization of splicing factors and poly(A)$^+$ RNA was simply the result of the temperature change, we examined and compared the localization of splicing factors and poly(A)$^+$ RNA in the parental cell line, BHK cells, grown both at the permissive and the restrictive temperatures. The comparison did not reveal detectable differences in the distribution of either splicing factors (our unpublished observation) or poly(A)$^+$ RNA (Figure 4, A and C) between cells grown at either temperature. This result demonstrated that the change in the organization of splicing factors and poly(A)$^+$ RNA observed in tsBN2 cells grown at the restrictive temperature was not merely a response to the temperature shift but was specifically due to the loss of RCC1 function.

We were also interested in determining whether the nucleolar structure was affected in RCC1$^-$ cells. The integrity of the nucleolus was examined with an antibody that specifically recognizes a nucleolar protein. The fibrillarin protein is a nucleolar-specific antigen that is associated with several small nucleolar RNAs (Tyc and Steitz, 1989). Immunostaining using an antibody that specifically recognizes the fibrillarin protein revealed little or no differences between cells that were grown at the permissive temperature (Figure 4B) and cells that were grown at the restrictive temperature (Figure 4D). This observation suggests that nucleolar structure remains largely unaffected in RCC1$^-$ cells at the light microscopic level, in spite of a dramatic alteration in the nucleoplasmic organization of splicing factors and poly(A)$^+$ RNA.

Transcription and Pre-mRNA Splicing Activities Are Not Drastically Altered within 6 h of RCC1 Inactivation

Previous studies have shown that inhibition of RNA transcription results in a similar reorganization of splicing factors and poly(A)$^+$ RNA in the cell nucleus (Spector *et al.*, 1993; Huang *et al.*, 1994). Although an earlier study by Nishimoto *et al.* (1978) showed that the bulk of RNA transcription was not significantly reduced within 12 h of RCC1 inactivation, it was important to directly examine the transcriptional activity in situ of cells that showed a reorganization of splicing factors and poly(A)$^+$ RNA upon RCC1 inactivation. Cells grown at both the permissive and restrictive temperature for 6 h were labeled with [^{3}H]uridine for 5 min (see MATERIALS AND METHODS). Analysis by autoradiography showed that the majority of cells grown at the restrictive temperature, without undergoing PCC, continued to transcribe RNA at a level comparable to that of cells grown at the permissive temperature (Figure 5). However, when a large number of cells was examined, we found that the transcriptional activity was more heterogeneous in cells grown at the restrictive temperature than in cells grown at the permissive temperature. This finding suggests that the redistribution of splicing factors and poly(A)$^+$ RNA is not the result of a drastic reduction of RNA transcription.

In addition, a previous report by O'Keefe *et al.* (1994) showed that inhibition of cellular splicing also induces a similar nuclear reorganization of splicing factors. To

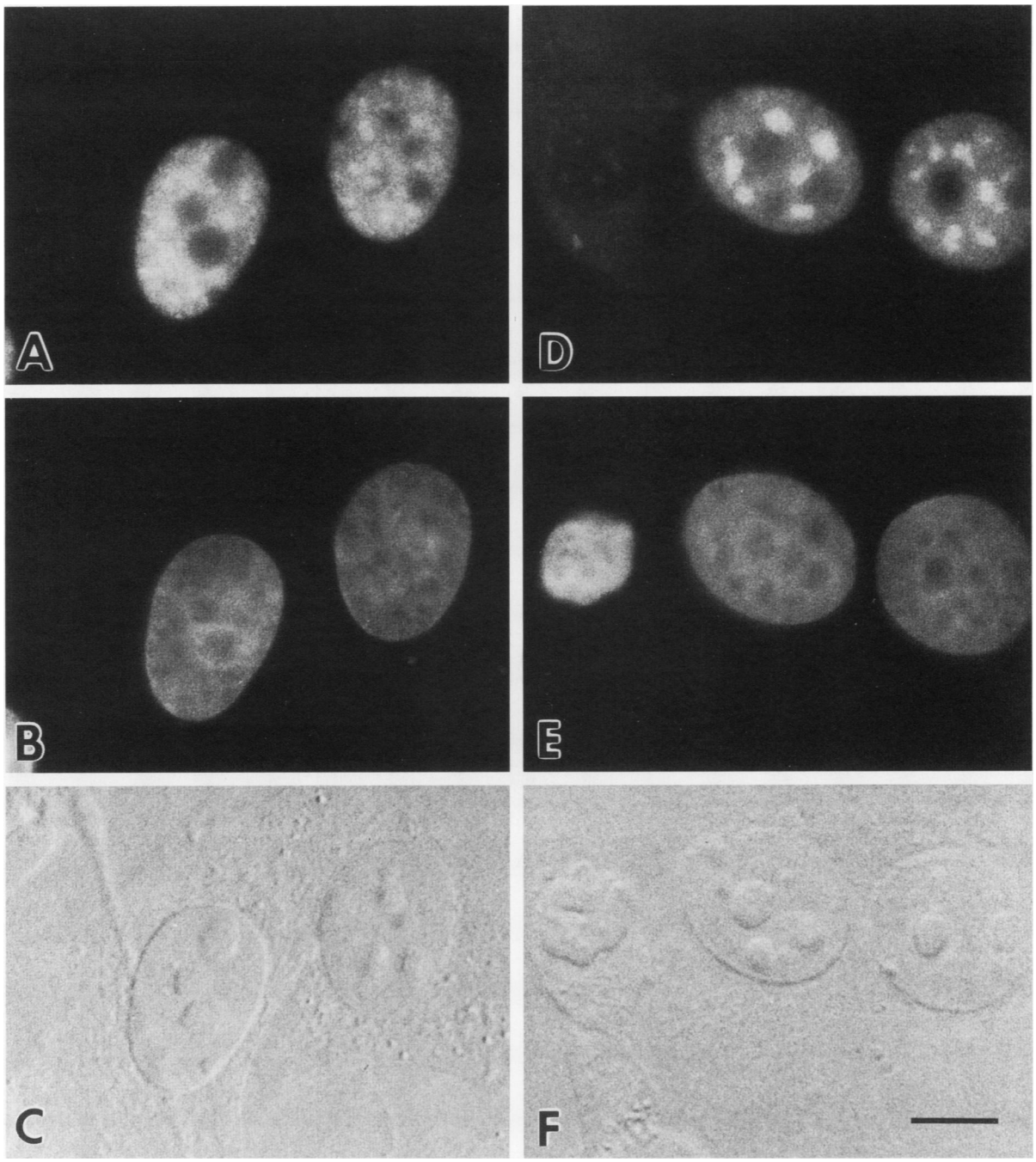

Figure 3. Redistribution of splicing factors and poly(A)$^+$ RNA in G_1 cells is not the result of premature chromatin condensation. Cells grown at the permissive temperature (A–C) or at the restrictive temperature (D–F) were simultaneously labeled for the localization of snRNPs (A and D) and DNA (B and E). The corresponding DIC images are C and F. Bar, 10 μm.

examine whether the pre-mRNA splicing machinery is significantly altered in RCC1$^-$ cells, we analyzed the ability of nuclear extracts from cells grown at either the permissive or the restrictive temperature to splice β-globin pre-mRNA in vitro (see MATERIALS AND METHODS). We found that nuclear extracts from cells grown at either the restrictive or the permissive temperature repeatedly spliced β-globin pre-mRNA into mRNA at a comparable efficiency (Figure 6, lanes 2 and 4). These experiments indicate that the splicing machinery in RCC1 inactivated cells is as functionally active as in their RCC1$^+$ counterpart.

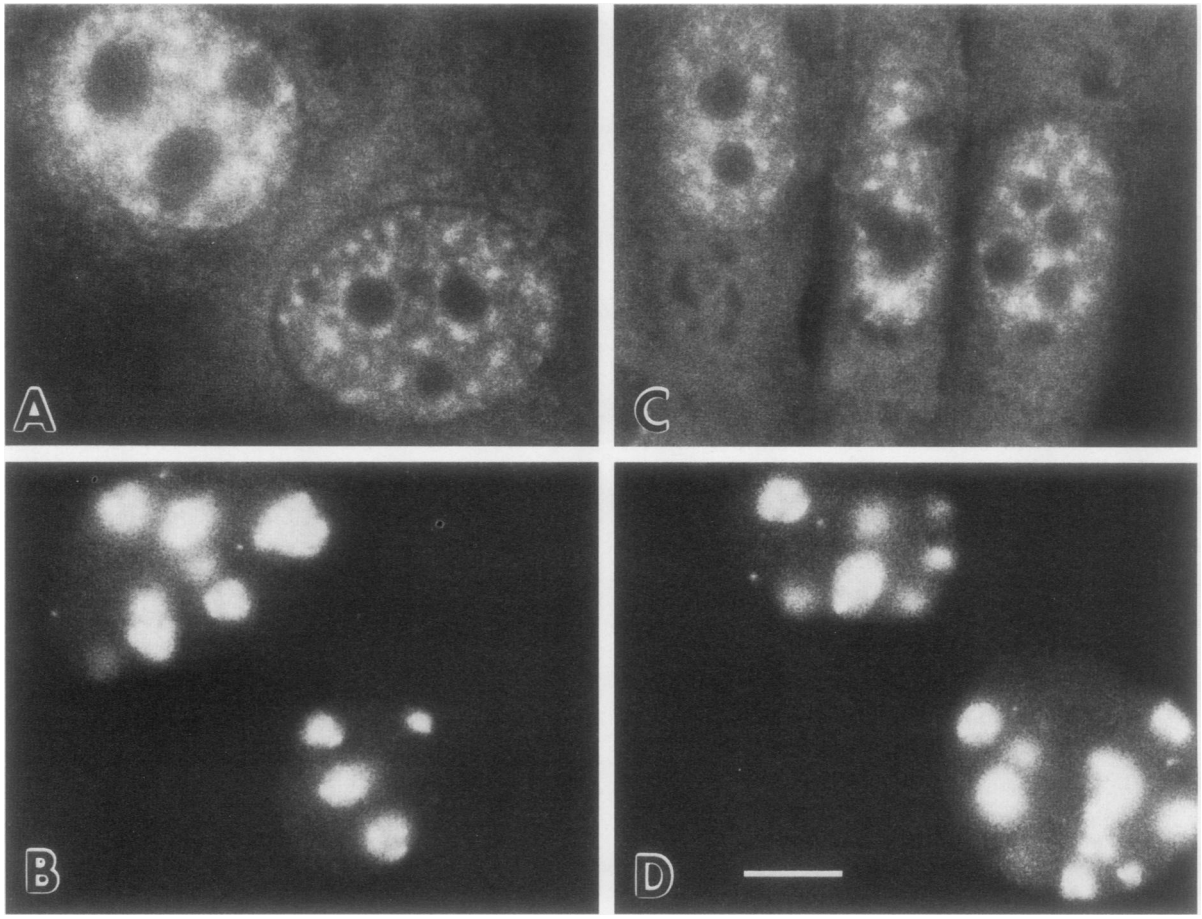

Figure 4. Redistribution of splicing factors and poly(A)$^+$ RNA is not due to the temperature change because the parental BHK cells, grown at either the permissive (A) or the restrictive (C) temperatures, show a similar speckled distribution of poly(A)$^+$ RNA. Furthermore, in spite of the changes in the distribution of splicing factors and poly(A)$^+$ RNA in the nucleoplasm, little alteration in nucleolar structure is observed between tsBN2 cells grown at the permissive (B) or the restrictive (D) temperatures. Nucleoli were immunolabeled with anti-fibrillarin antibody. Bar, 10 μm.

Electron Microscopic Analysis of tsBN2 Cell Nuclei in the Absence of RCC1 Function

To evaluate the nature of the larger clusters enriched in splicing factors and poly(A)$^+$ RNA, as well as other possible changes in nuclear structure in RCC1$^-$ cells at a higher resolution, we compared the ultrastructure of tsBN2 cells grown at either the permissive or the restrictive temperatures. Electron micrographs of cells grown at the restrictive temperature revealed that the large clusters that contain splicing factors and poly(A)$^+$ RNA at the light microscopic level correspond to fused round interchromatin granule clusters (Figures 7B, arrowheads, and 8A). Interchromatin granule clusters normally are difficult to observe in uranyl acetate- and lead citrate-stained sections as seen in cells grown at the permissive temperature (Figure 7A). In addition to the fused interchromatin granule clusters, small electron-dense dots were also observed to be accumulated in the fused interchromatin granule clusters and dispersed in the nucleoplasm (Figures 7B and 8A, arrowheads). These dots measured approximately 80 nm in diameter. When thin sections (80–100 nm in thickness) were probed with anti-SC35 antibody, some of these structures were labeled, suggesting that they contain splicing factors (Figure 8B, arrowheads). RNase A and DNase I treatment prior to the plastic embedding did not affect these structures, suggesting that their integrity may not be dependent on the association with RNA or DNA (our unpublished observations). Interchromatin granule clusters show a similar resistance to DNase I and RNase A digestions in the absence of protease (for a review, see Fakan and Puvion, 1980). The nature of these electron-dense dots formed in RCC1$^-$ cells remains unclear. Although interchromatin granule clusters are significantly enlarged, perichromatin fibrils are still visible (Figure 8B, arrows). In spite of the massive change in nucleoplasmic organization, few

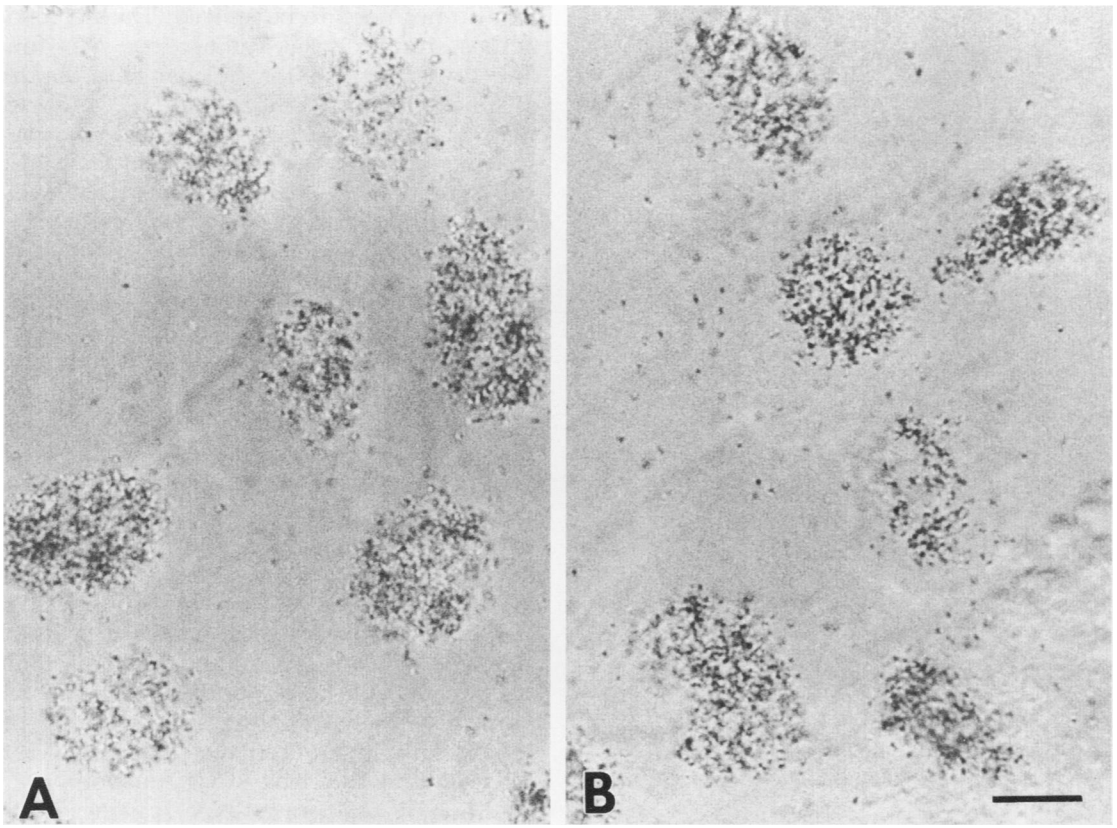

Figure 5. Transcriptional activity in RCC1$^-$ cells is comparable to that of RCC1$^+$ cells at 6 h after the RCC1 inactivation. The autoradiographic grains represent the sites of transcription after incorporation of [^{3}H]uridine for 5 min in cells grown at the permissive temperature (A) and cells grown at the restrictive temperature (B). Bar, 10 μm.

differences were observed in nucleolar structure. Fibrillar centers were clearly visible, although their electron density appeared to be variable from cell to cell at both the permissive and the restrictive temperatures. In addition, gross changes in the nuclear envelope or state of chromatin compacting were not observed (Figure 7B).

When S-phase cells were grown at the restrictive temperature, premature chromatin condensation pushed the cell into a premature cell division before the completion of DNA replication. The newly divided cells contained smaller nuclei with condensed chromatin (Figure 7C). In these nuclei, condensed chromatin filled up most of the nuclear volume. Much of the nuclear structure such as, nucleoli, interchromatin granule clusters, and perichromatin fibrils, was not easily distinguishable. However, the nuclear envelope did not exhibit gross alterations (Figure 7C).

DISCUSSION

We have examined the nuclear organization of pre-mRNA splicing factors and poly(A)$^+$ RNA in tsBN2 cells when RCC1 function is lost due to a temperature-sensitive mutation. We found that splicing factors and poly(A)$^+$ RNA are redistributed into larger but fewer interchromatin granule clusters when cells are grown at the restrictive temperature. Such alteration is not the result of DNA condensation or the temperature shift. Bulk reduction in RNA transcription and significant alterations in the pre-mRNA splicing machinery were not observed in these cells. In addition, small electron-dense dot-like structures measuring approximately 80 nm in diameter were observed in interchromatin granule clusters or dispersed in the nucleoplasm of RCC1$^-$ cells. However, the nature of these structures is unclear. In spite of the drastic change in the organization of the nucleoplasm, significant alterations in nucleolar structure were not observed by either light or electron microscopic examination.

Alteration in the Nuclear Organization of Splicing Factors in RCC1$^-$ Cells

The loss of RCC1 function causes a redistribution of splicing factors in G_1 cells, suggesting that the RCC1

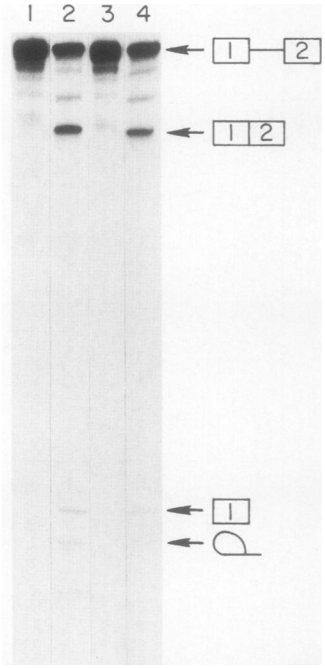

Figure 6. Splicing machinery remains functional in RCC1$^-$ cells at 6 h after RCC1 inactivation. Nuclear extracts from cells grown at the restrictive temperature (lanes 1 and 2) and the permissive temperature (lanes 3 and 4) splice β-globin pre-mRNA at a comparable efficiency.

protein is directly or indirectly responsible for the maintenance of the speckled organization of splicing factors under normal circumstances. A similar reorganization of splicing factors in the cell nucleus has been reported in several recent studies. When transcription by RNA polymerase II is inhibited by the addition of α-amanitin, splicing factors along with poly(A)$^+$ RNA are reorganized into larger and fewer clusters that correspond to fused interchromatin granule clusters at the electron microscopic level (Spector *et al.*, 1993; Huang *et al.*, 1994). A recent study has suggested that serine/threonine phosphatase 1 may be required for this reorganization (Misteli and Spector, 1996). The rationale behind the change in the distribution of splicing factors upon the inhibition of RNA synthesis is thought to be that splicing factors return to and/or stay within interchromatin granule clusters because substrates are not available to splice due to the inhibition of RNA synthesis. In a second study, microinjection into cells of oligonucleotides or antibodies that inhibit pre-mRNA splicing in vitro showed that splicing factors are reorganized into fewer and larger interchromatin granule clusters (O'Keefe *et al.*, 1994). A similar rearrangement of splicing factors has also been observed in cells infected with the herpes simplex virus (Martin *et al.*, 1987; Phelan *et al.*, 1993). In this case, the virus produces a large population of RNAs that do not need to be spliced. The expression of the herpes simplex virus-1 IE63 gene was found to be directly responsible for the change in the distribution of splicing factors (Phelan *et al.*, 1993). These observations suggest that the typical speckled organization of splicing factors is dependent upon the transcriptional and pre-mRNA splicing activities of the cell. The redistribution of splicing factors in response to the loss of RCC1 function resembles the change observed in the above studies. However, our finding as well as previous results (Nishimoto *et al.*, 1978) demonstrated that the bulk of RNA transcription is not significantly reduced within 6 h of inactivation of the RCC1 protein. Thus, the reorganization of splicing factors cannot be explained by the inhibition of overall RNA synthesis, although this does not exclude the possibility that inhibition of the synthesis of one or more specific RNAs may be responsible for the observed structural reorganization. Another explanation for the reorganization of splicing factors is that the loss of RCC1 function affects pre-mRNA splicing activity. However, nuclear extracts prepared from tsBN2 cells at both the permissive and the restrictive temperatures are equally able to support the splicing of β-globin pre-mRNA in vitro, suggesting that the pre-mRNA splicing machinery remains intact and functionally active in cells that lack RCC1 function.

Although the pre-mRNA splicing machinery in RCC1$^-$ cells appears to be as competent as that in RCC1$^+$ cells, pre-mRNA splicing may still be affected in these cells. Studies in the yeast system have shown that a mutation in prp20 generates mRNA species with abnormal lengths (Aebi *et al.*, 1990). In addition, Forrester *et al.* (1992) have found that the prp20 mutant is defective in the 3′ end cleavage of several different mRNAs and in the processing of RNAs at their normal polyadenylation sites. Such abnormalities in the length or 3′ end processing of pre-mRNAs may affect their availability to be spliced. Under these conditions, one would expect that splicing factors remain at or return to storage/reassembly sites (interchromatin granule clusters).

Alternatively, it is also possible that a factor(s) that participates in maintaining an underlying nuclear structure is the direct or indirect target of RCC1 regulation. An alteration in nuclear structure could prevent the functional organization of splicing factors that in turn could affect RNA processing. One of our

Figure 7 (facing page). Interchromatin granule clusters become larger in size and fewer in number when tsBN2 cells were grown at the restrictive temperature (B, arrowheads), as compared with cells grown at the permissive temperature (A). When S-phase cells (C) were grown at the restrictive temperature, condensed chromatin occupied the majority of the nuclear volume, and nuclei were much smaller. Bar, 1 μm.

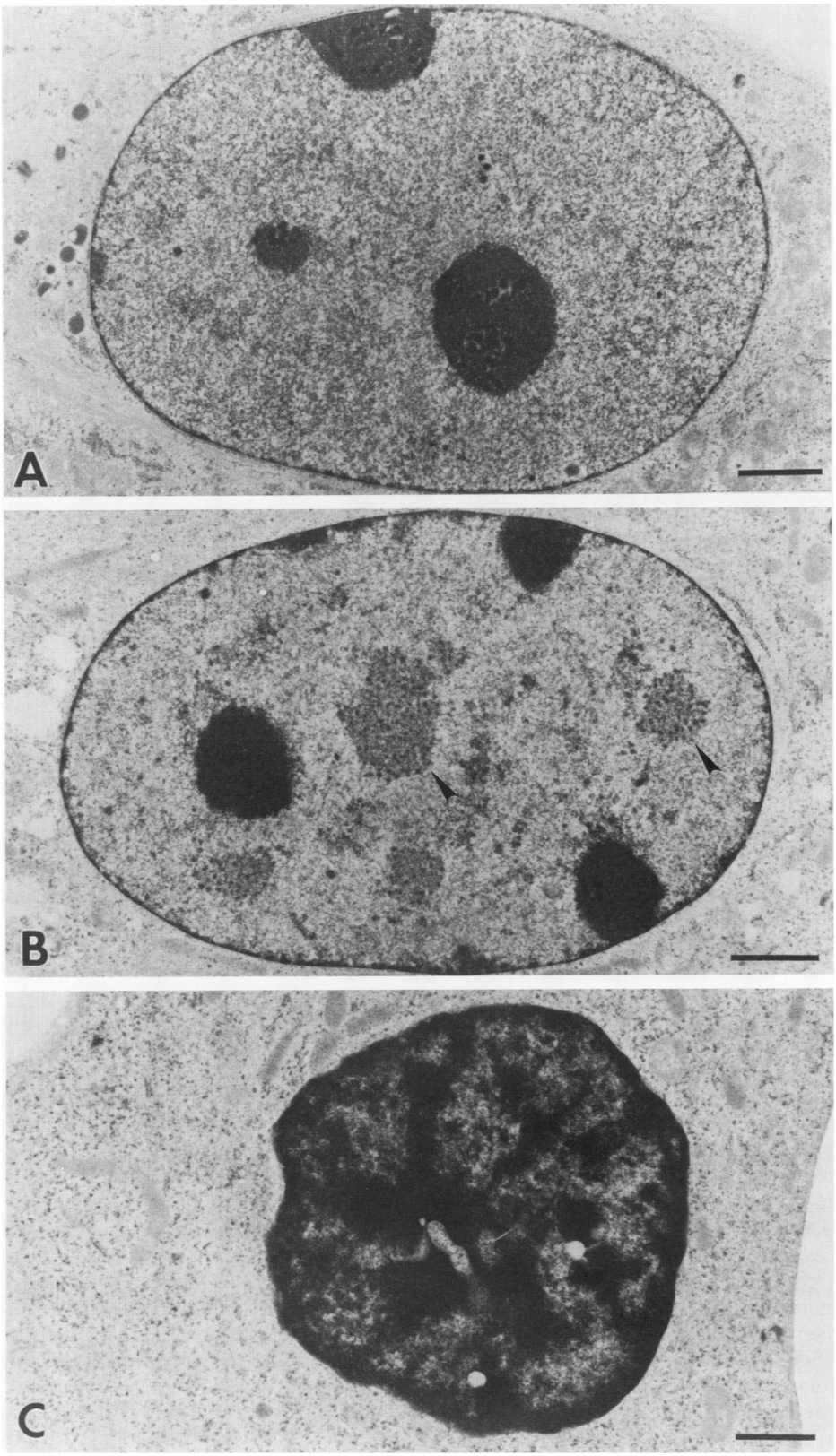

Figure 7.

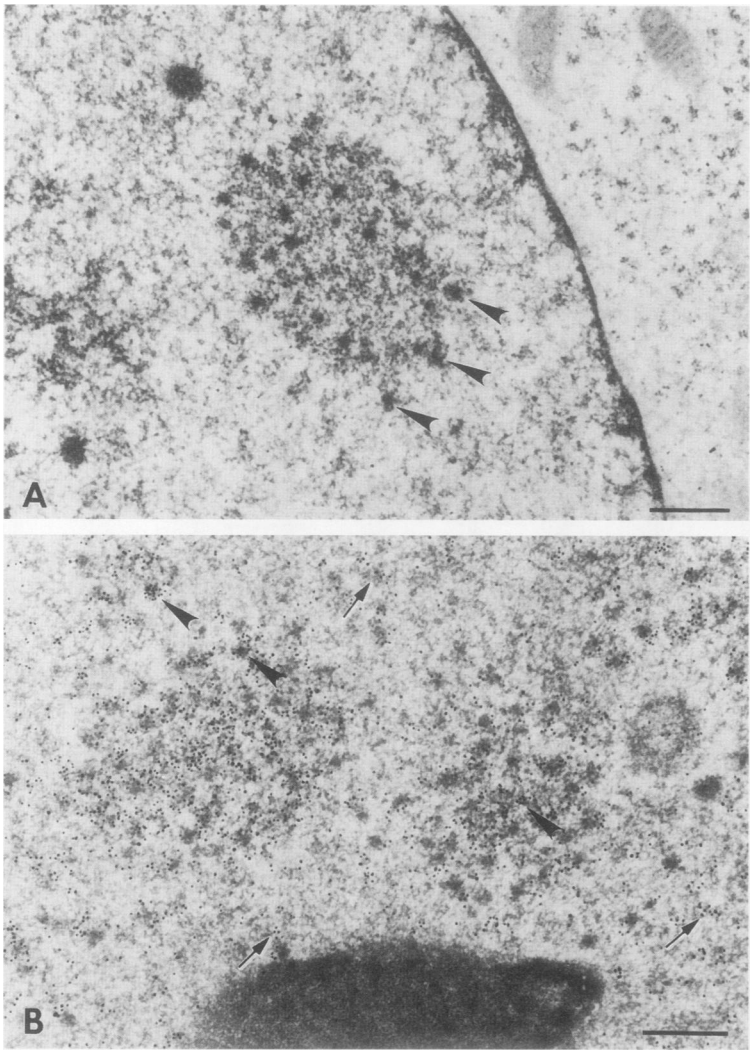

Figure 8. In RCC1$^-$ nuclei, 80-nm dot-like structures (A, arrowheads) are accumulated within interchromatin granule clusters as well as being dispersed in the nucleoplasm. When sections were immunogold labeled with anti-SC35 antibody, many of these structures were found to contain the SC35 splicing factor (B, arrowheads). Although interchromatin granule clusters have changed significantly, perichromatin fibrils can still be observed (arrows). Bar, 0.5 μm.

observations is consistent with this possibility. Normally, the number of interchromatin granule clusters ranges from 20 to 50 per mammalian cell nucleus. But, in RCC1$^-$ cells, the number decreases significantly to 4–10 per nucleus, with much larger clusters. Such an alteration suggests that fusion between multiple small clusters takes place when the RCC1 protein is inactivated. The fusion between interchromatin granule clusters represents a dramatic intranuclear movement or architectural rearrangement. However, it is not clear whether the change in nuclear structure is the primary result due to the loss of RCC1 function or is a secondary response to alterations of other nuclear functions. Nevertheless, studies have shown that some of the ras-related small GTP-binding proteins such as rho and rac regulate the organization of the actin cytoskeleton (for review, see Hall, 1992). Since the RCC1 protein functions through ran, a nuclear ras-related GTPase, it is possible that ran is also involved in regulating the organization of the nuclear skeleton. If this is the case, the changes observed in nuclear structure of RCC1$^-$ cells may be an event that occurs before or is responsible for the defect in other nuclear functions. In addition, studies have shown that both the wild-type RCC1 and Ran/TC4 proteins are essential for the formation and the maintenance of the structural integrity of nuclei (Kornbluth *et al.*, 1994; Dasso *et al.*, 1994; Demeter *et al.*, 1995). In an in vitro nuclear assembly assay using *Xenopus* egg extracts, Kornbluth *et al.* (1994) and Dasso *et al.* (1994) found that a mutant TC4 protein that was defective in GTP binding suppressed nuclear assembly and prevented DNA replication from occurring. In the fission yeast system, a mutation in pim1 (RCC1-related protein) leads to fragmentation of the nuclear envelope (Demeter *et al.*, 1995). However, it is interesting that a gross change in the nuclear envelope was not observed in RCC1$^-$ tsBN2 cells, suggesting that RCC1 and RCC1-

related proteins may play different roles in mammalian cells and in fission yeast.

Alteration in the Nuclear Localization of Poly(A)$^+$ RNA in RCC1$^-$ Cells

When cells are grown at the restrictive temperature and the RCC1 protein is inactivated, poly(A)$^+$ RNA is also redistributed into larger fused interchromatin granule clusters. Poly(A)$^+$ RNA in the nucleus consists of at least three groups of RNAs including pre-mRNA, mRNA, and nuclear RNAs, which may not serve as mRNAs (Brockdorff *et al.*, 1992; Brown *et al.*, 1992; Hogan *et al.*, 1994; Huang *et al.*, 1994). Normally, nuclear poly(A)$^+$ RNA is distributed in a speckled nuclear pattern in addition to being diffusely distributed throughout the nucleoplasm and cytoplasm. The speckled distribution of poly(A)$^+$ RNA corresponds to the localization of splicing factors that includes both interchromatin granule clusters and perichromatin fibrils (Carter *et al.*, 1991; Huang *et al.*, 1994). The poly(A)$^+$ RNAs in many of the interchromatin granule clusters are most likely noncoding RNAs as these structures do not contain newly synthesized RNA as demonstrated by [^{3}H]uridine incorporation experiments (Fakan and Bernhard, 1971; Fakan and Nobis, 1978; Fakan, 1994), and these RNAs cannot be chased out of the nuclei during transcription inhibition (Huang *et al.*, 1994). The RNAs present in these clusters may play structural and/or regulatory roles in the cell nucleus. In contrast, RNAs on the surface of interchromatin granule clusters, in perichromatin fibrils, and diffusely distributed throughout the nucleus may represent newly synthesized pre-mRNA and mRNA. When RCC1 function is lost, poly(A)$^+$ RNA along with splicing factors are rearranged and colocalized into fused interchromatin granule clusters in addition to being diffusely distributed in the nucleoplasm. The diffuse staining in the nucleoplasm observed at the light microscopic level and perichromatin fibrils detected at the electron microscopic level may partially represent the sites of nascent RNA synthesis, since our finding and an earlier study showed that transcription in RCC1$^-$ cells is not significantly impaired within 6 h of RCC1 inactivation (Nishimoto *et al.*, 1978). Other diffusely distributed RNA may represent the accumulation of RNA that failed to export into the cytoplasm (Amberg *et al.*, 1993; Kakowaki *et al.*, 1993; Schlenstedt *et al.*, 1995). As to the large accumulation of RNA in fused interchromatin granule clusters, some of these RNAs could normally be components of these structures, whereas others may account for the newly synthesized RNAs that do not undergo the normal post-transcriptional processing and export, because previous studies have shown these functions to be altered in RCC1$^-$ cells (Aebi *et al.*, 1990; Forrester *et al.*, 1992; Amberg *et al.*, 1993; Kakowaki *et al.*, 1993; Cheng *et al.*, 1995; Schlenstedt *et al.*, 1995). It is particularly interesting to note the possibility that the 80-nm dot-like particles may represent pre- and/or messenger ribonucleoprotein particles that accumulate in the nuclei at the restrictive temperature. However, it is unclear how the loss of RCC1 function leads to the redistribution of nuclear poly(A)$^+$ RNA. Either the alterations in RNA metabolism, including post-transcriptional processing and transport, or alterations in nuclear architecture could be the direct or indirect cause of the redistribution. Future studies are needed to elucidate the link between RCC1/ran and the reorganization of nuclear components.

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