Spliced Exons of Adenovirus Late RNAs Colocalize with snRNP in a Specific Nuclear Domain

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Abstract. Posttranscriptional steps in the production of mRNA include well characterized polyadenylation and splicing reactions, but it is also necessary to understand how RNA is transported within the nucleus from the site of its transcription to the nuclear pore, where it is translocated to the cytoplasmic compartment. Determining the localization of RNA within the nucleus is an important aspect of understanding RNA production and may provide clues for investigating the trafficking of RNA within the nucleus and the mechanism for its export to the cytoplasm. We have previously shown that late phase adenovirus-infected cells contain large clusters of snRNP and non-snRNP splicing factors; the presence of these structures is correlated with high levels of viral late gene transcription. The snRNP clusters correspond to enlarged interchromatin granules present in late phase infected cells. Here we show that polyadenylated RNA and spliced tripartite leader exons from the viral major late transcription unit are present in these same late phase snRNP-containing structures. We find that the majority of the steady state viral RNA present in the nucleus is spliced at the tripartite leader exons. Tripartite leader exons are efficiently exported from the nucleus at a time when we detect their accumulation in interchromatin granule clusters. Since the enlarged interchromatin granules contain spliced and polyadenylated RNA, we suggest that viral RNA may accumulate in this late phase structure during an intranuclear step in RNA transport.

THE production of eukaryotic mRNAs can be considered in several steps; these include transcription, polyadenylation, splicing, and export of mRNA from the nucleus to the cytoplasm. Export involves movement of the RNA within the nucleus from the site of transcription to the nuclear pore, followed by transport of the message through the nuclear pore and into the cytoplasm. Although it is convenient to think of these posttranscriptional processes as separate steps, they are likely to be inter-related during the production of RNA in vivo. All of these reactions occur within the structural framework of the nucleus; thus the nuclear organization of gene expression activities has recently received considerable attention (for reviews see Carter, 1994; Wansink et al., 1994; Bridge et al., 1995).

The nucleus has a high degree of organization with the nucleolus as the most obvious and well characterized nuclear compartment (Scheer et al., 1993). Chromatin in various degrees of condensation occupies much of the nuclear interior. Interchromatin regions contain several characteristic structures which have been visualized by electron microscopy (for review see Fakan, 1994). These include perichromatin fibrils, interchromatin granules, and coiled bodies. Perichromatin fibrils are strongly labeled after short pulses of [^{3}H]uridine indicating that transcription occurs at these structures. Perichromatin fibrils are thought to be the in situ forms of newly synthesized hnRNA. Interchromatin granules are not strongly labeled by a pulse of [^{3}H]uridine and are therefore not likely to be locations of transcription, but several studies have shown that polyadenylated RNA is present in these structures (Carter et al., 1991, 1993; Visa et al., 1993). SnRNP and several non-snRNP splicing factors accumulate at both perichromatin fibrils and interchromatin granules, raising the possibility that splicing of precursor RNA may occur at these locations. Previous work suggests that some splicing of transcripts occurs at the site of transcription (Beyer and Osheim, 1988; Zhang et al., 1994; Baurén and Wieslander, 1994; Baurén et al., 1996; Huang and Spector, 1996); but splicing has also been observed to occur posttranscriptionally (Baurén and Wieslander, 1994). Although splicing factors are present in the coiled body, this structure is not thought to be directly involved in the splicing of RNA, but may rather be involved in some aspect of the trafficking of splicing factors (Lamond and Carmo-Fonseca, 1993).

We are studying the organization of gene expression ac-

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tivities in the nucleus using adenovirus (ad)[1]-infected cells as a model system. We have focused our initial studies on the distribution of splicing factors during ad infection. The infectious program of human ads types 2 and 5 consists of an early program of gene expression which is followed by DNA replication. DNA replication is required for the subsequent expression of the viral late genes. During the viral late phase splicing factors accumulate in large centers which we have referred to as "late phase snRNP clusters" (Bridge et al., 1993, 1995). We and others have provided evidence that these centers correspond to enlarged interchromatin granules present during the late phase of infection (Bridge et al., 1993; Puvion-Dutilleul et al., 1994). Both snRNP and SR splicing factors are present in these late phase clusters. The presence of splicing factors in snRNP clusters is well correlated with the production of viral late mRNA and proteins (Bridge et al., 1993, 1995). We have found that maximum transcription from the viral major late transcription unit (MLTU) occurs at a time when splicing factors are present in snRNP clusters in >90% of the cells in the culture (Bridge et al., 1995). Viral RNA and polyadenylated RNA have been detected in the late phase interchromatin granules that correspond to the snRNP clusters (Puvion-Dutilleul et al., 1994). These data suggest a role for the enlarged interchromatin granules in the production of viral RNA.

The identity and the significance of the RNA species present in the interchromatin granule structure is the subject of current investigation and debate. Huang et al. (1994) find that polyadenylated RNA remains associated with the interchromatin granule after inhibition of transcription, and have suggested that this may be a stable population of polyadenylated RNA that is not mRNA. Recently Xing et al. (1995) have reported that specific cellular RNAs are spatially associated with the interchromatin granule compartment; the collagen Iα1 RNA is observed to extend into this compartment from the transcribing gene which is located at its periphery. Ad-infected cells can provide a useful model for studying the significance of the interchromatin granule in RNA production since these structures are enlarged during the late phase when the infected cell produces large quantities of RNA from the viral MLTU (Bridge et al., 1993, 1995; Puvion-Dutilleul et al., 1994).

Here we study the identity and structure of the viral RNA present in the late phase snRNP-containing interchromatin granule clusters using in situ hybridization techniques. Most ad late messages are produced from the viral MLTU (for review see Sharp, 1984). The primary transcript is differentially polyadenylated and spliced to a variety of different mRNAs that encode most of the viral late protein products. The mRNAs produced from the MLTU have three exons of the tripartite leader sequence spliced to the 5′ end of each message body. We find that late phase snRNP clusters contain RNA from the MLTU. Oligonucleotide probes complementary to spliced RNA sequences from the tripartite leader show that spliced forms of viral late transcripts are present in the snRNP clusters. Oligo dT probes demonstrate that polyadenylated RNA is localized in the clusters. These data show that detectable levels of the RNA in the interchromatin granule clusters have undergone mRNA processing modifications at either or both the 5′ and 3′ ends of the message. Since these processing modifications are observed in cytoplasmic viral mRNAs, we suggest that the viral RNA detected in the late phase interchromatin granules may be nuclear RNA sequences that are in transit to the cytoplasmic compartment.

1. *Abbreviations used in this paper*: ad, adenovirus; FFU, focus forming unit; h p.i, hour postinfection; MLTU, major late transcription unit; n, nucleotide.

Materials and Methods

Cell Culture and Viral Infections

HeLa cell monolayer cultures were maintained in Dulbecco's modified medium containing 10% FCS. Cells were infected with ad2 at a multiplicity of infection (MOI) of 20 focus forming units (FFU)/cell. Virus titers, expressed as FFU/ml, were determined as described (Philipson, 1961).

Fixation, In Situ Hybridization, and Immunofluorescence Staining of Cells

Ad2- and mock-infected cells grown on glass coverslips were fixed at 18 h postinfection (h p.i.). Coverslips with attached cells were washed twice in PBS, extracted for 2–3 min on ice with 0.5% Triton X-100 in 100 mM NaCl, 10 mM Pipes, pH 6.8, 3 mM $MgCl_2$, 1 mM EGTA, 0.3 M sucrose (CSK buffer), and then fixed in 3.7% paraformaldehyde in CSK buffer for 10 min at room temperature. In the experiment shown in Fig. 2 *A*, the cells were washed twice in PBS and then fixed in 3.7% paraformaldehyde in CSK buffer for 10 min at room temperature. After fixation the cells were extracted with 0.1% sodium dodecyl sulfate (SDS), 100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 12.5 mM EDTA for 5 min at room temperature. This protocol was used to preserve the cytoplasmic RNA signal which was lost following pre-extraction in 0.5% Triton X-100 in CSK buffer.

In situ hybridizations and detection of biotin-labeled probes were done essentially as described (Pombo et al., 1994). However, it was necessary to adjust the hybridization and washing conditions for each probe to obtain the least background staining in uninfected cells. Our standard protocol was as follows. Fixed cells on coverslips were washed twice with 2× SSC. 25–100 ng of biotin- or digoxigenin-labeled probes were dissolved in 8 μl of hybridization buffer consisting of 50% formamide, 2× SSC, 1 μg/μl *E.coli* tRNA, and 5% Dextran sulfate. The probes were denatured for 5 min at 65°C and chilled on ice. Coverslips containing fixed cells were incubated with 8 μl of hybridization solution at 37°C for 1–4 h. The fixed cells were not denatured before hybridization. After the hybridization, cells were washed in 2× SSC for 3 times 15 min at 37°C, followed by a wash in 1× SSC for 15 min at room temperature. Probe SJ3 was processed as described above except that the final wash was done at 45°C. With probe SJ2, the hybridization was at room temperature and the final wash following hybridization was at 45°C. With oligo dT, hybridization was done at 42°C. Probes IN1, USJ5, and USJ6 were hybridized in buffer containing 25% formamide and 10% dextran sulfate, and the hybridization was done at 42°C.

For detection of biotin-labeled probes cells were washed with 20 mM Hepes, pH 7.5, 150 mM KCl, 0.05% Tween-20 (avidin wash buffer) and incubated for 30 min with 7 μl of 12.5 μg/ml extravidin (Sigma) in 20 mM Hepes, 150 mM KCl, 0.5 mM DTT, 1% BSA. Cells were then washed for 10 min in avidin wash buffer, and then for two times five min in 4× SSC. Cells were mounted in Vectashield (Vector Labs, Inc., Burlingame, CA) or immunolabeled to detect snRNP.

Immunostaining of digoxigenin-labeled probes or snRNP was done following the in situ hybridization washes, or the biotin detection procedure, respectively. Antibodies were incubated with the cells for 30 min in a solution of 100 mM Tris-HCl, pH7.5, 150 mM NaCl, 0.5% Blocking Reagent (Boehringer Mannheim, Indianapolis, IN). Washes were done in 4× SSC instead of PBS. Monoclonal antibody Y12 (Lerner et al., 1981) was used to detect snRNP. Digoxigenin was detected with commercial antibodies from Boehringer Mannheim. Secondary antibodies were from Southern Biochemicals (Birmingham, AL).

In some experiments cells on coverslips were incubated with 8 μl of 100 μg/ml RNAse A in 2× SSC, or 8 μl of 100 U/ml RNase free DNase I

(Promega) in 40 mM Tris-HCl, pH 7.9, 6 mM $MgCl_2$ for 45 min in a moist chamber at 37°C following fixation. Cells were washed for three times 10 min in 2× SSC and then hybridized as described above. After the in situ hybridization washes cells from some experiments were incubated with 8 μl of 75 U/ml RNAse H (Amersham) in 40 mM Tris-HCl 7.5, 4 mM $MgCl_2$, 1 mM DTT, 4% glycerol, 30 μg/ml BSA, 100 mM KCl, for 45 min in a moist chamber at 37°C. Cells were washed for three times 10 min in 4× SSC and biotin-labeled probes were detected as described above.

Conventional fluorescence microscopy (Figs. 2, 3, and 4) was performed with a Nikon Optiphot-2 microscope using a 100× objective. Cells were photographed with Kodak Tmax 400 film using the Nikon UFX-DX camera system (Figs. 2 and 3). Alternatively, cells were visualized with a Meridian cooled CCD camera system from Colorado Video Inc. (Boulder, CO) (Fig. 4). Confocal microscopy (Fig. 5) was performed with a multiprobe 2000 instrument (Molecular Dynamics, Sunnyvale , CA) equipped with an argon/krypton laser scanner using wavelengths of 488 and 568 to detect fluorescence from FITC and Texas red, respectively.

All oligonucleotides used in this work were purchased commercially from Operon or from Pharmacia. The sequence of the oligonucleotide probes used for the in situ hybridizations were as follows. SJ1: 5'XCAACCGCGAGCCCAACAGCTG3', SJ1(11b):5'XCCCAACAGCTG3', SJ2:5'XGGCGGAGTACCGTTCGGAGG3', SJ3:5'XCAGCAGCTCCTCTTGCGACTG3', oligo(dT);5'XTTTTTTTTTTTTTTTTTTTTT3',USJ55'YGCTCAGCCTACCTTGCGACTG3', USJ6:5'YCAGCAGCTCCTCTGGCGGCGAC3', IN1:5'YGTCTTTTCTGACCAGATGGACG3'. X corresponds to biotin and Y to digoxigenin. PCR primers used to generate PCRex were 5'CTCTCTTCCGCATCGCTGTC3' and 5'CTTGCGACTGTGACTGGTTAG3'. PCR primers used to generate PCRin were 5'GAGTACTCCCTCTCAAAAGCG3' and 5'CGCGGCCAAGGAGCGCGCCG3'. PCR reactions were performed with biotin-16-dUTP (Boehringer Mannheim) present in the nucleotide mixture to label the PCR product. The PCR reactions contained 134 μM dTTP, 66 μM Biotin-16-dUTP, and 200 μM dATP, dGTP, and dCTP. Probes generated by PCR were purified by G-50 Sephadex spin column chromatography before their use for in situ hybridizations and the concentrations estimated by gel electrophoresis.

Preparation of Nuclear and Cytoplasmic RNA

Total RNA was prepared from the cytoplasms and nuclei of infected cells as described (Sambrook et al., 1989; Smiley et al., 1995). Except where indicated, all procedures were carried out on ice. 4–6 × 10^6 infected or uninfected cells were washed twice with ice cold PBS, scraped into 1 ml of PBS and transferred to a microcentrifuge tube. Cells were pelleted by centrifugation and resuspended in 0.4 ml 10 mM Tris-HCl, pH 7.5, 140 mM NaCl, 1.5 mM $MgCl_2$, 0.5% NP-40. Cells were incubated for 5 min, and the nuclei were then pelleted by centrifugation in a microcentrifuge. The supernatant was removed for the cytoplasmic fraction. This fraction was centrifuged at top speed for greater than 1 min to remove any residual nuclei and the cytoplasm was transferred to a new tube. Nuclei were washed by resuspending in 0.4 ml 10 mM Tris-HCl, pH7.5, 140 mM NaCl, 1.5 mM $MgCl_2$, 1.0% NP-40, pelleted by centrifugation and resuspended in 0.4 ml of the same buffer. 5 U of RNAse free DNAse I (Promega, Madison, WI) was added to the nuclei and they were incubated for 5 min at 37°C. SDS and proteinase K were then added to both the nuclear and cytoplasmic fractions to a final concentration of 1.0%, and 1 mg/ml, respectively, and incubated at 37°C for 30 min. Nucleic acids were precipitated in ethanol following organic extraction. Nucleic acids were dissolved in 200 μl of 40 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, containing 75 U/ml RNAse free DNase I (nuclear fraction) and 25 U/ml (cytoplasmic fraction) and incubated for 45 min at 37°C. After organic extraction RNA was precipitated and stored in ethanol.

S1 Nuclease Quantitation of Viral RNA

Cytoplasmic and nuclear RNA was prepared as described above. The level of spliced and unspliced RNA from the tripartite leader was determined by S1 nuclease protection as described (Sambrook et al., 1989; Bridge et al., 1991), using 2–10 μg of nuclear or cytoplasmic RNA prepared at various times after infection. Equal amounts of RNA from uninfected cells were included as controls. We used 5′ end labeled synthetic oligonucleotide probes to detect RNA from the tripartite leader. These probes overlapped the 5′ end of exon 2 and the 5′ end of exon 3 of the tripartite leader. The sequences of these probes were 5'GACCGCGAAGAGTTTGTCCTCAACCGCGAGCTGTGGAAAAAAAAGGGACAGACCCAGGGATTGGC3', and 5'GCGGACTCGCTCAGGTCCCTCGGTGGCGGAGTACCTACACAACAATTGTTGAGATGCCCCAGGGATTGGC3', respectively. The 3′ end of each probe contained extra bases from the bacterial chloramphenicol gene so that full-length probes could be distinguished from the portion of the probe protected by unspliced tripartite leader RNA. After digestion with S1 nuclease, samples were fractionated on 10% acrylamide 7 M urea gels. The gels were then analyzed by phosphorimaging using a 400S instrument (Molecular Dynamics).

[^{3}H]Uridine Incorporation into Nuclear and Cytoplasmic RNA

Infected cells at 18 h p.i. or uninfected cells were incubated with media containing 0.2 mCi/ml [^{3}H]uridine and cold uridine to a final concentration of 14 μM for 15, 30, 60, 90, and 120 min. Nuclear and cytoplasmic RNA was prepared as described above except that following digestion with RNase free DNase, the samples were subjected to G-50 Sephadex spin column chromatography to remove the unincorporated [^{3}H]uridine label. Hybridization of labeled RNA to immobilized DNAs on filters and subsequent washing and RNAse treatment was as described (Ausubel et al., 1989) for hybridization of labeled RNA to DNA containing filters following nuclear run on analysis. However, the labeled RNA was not degraded by base treatment in our experiments. The spliced tripartite leader sequence from a cDNA clone (ptripCat, Nordqvist and Akusjärvi, 1990) was subcloned into pGem T vector following PCR amplification to create plasmid p31, containing the 201n spliced ad tripartite leader. Plasmid pDX589 containing sequences corresponding to ad late region 3 has been previously described (Bridge et al., 1995). Labeled RNA prepared from approximately 6 × 10^6 cells was hybridized to 20 μg of p31 DNA and 10 μg of pDX589 immobilized on filters. An equal amount of pGem 11 DNA without any insert was used as a control to determine background hybridization. RNA hybridizing to the filters was detected by scintillation spectrometry.

Results

Ad RNA from the MLTU Is Present in Discrete Nuclear Centers

We have determined the location of ad RNA in late

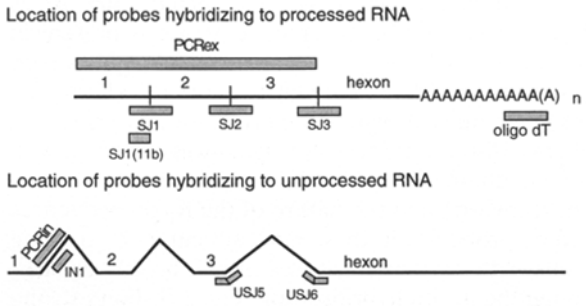


Figure 1. The location of probes used for in situ hybridizations relative to their target sequences in spliced and unspliced forms of viral late RNA. Synthetic oligonucleotide probes were 20–22 n in length and were labeled with either biotin or digoxigenin for detection after in situ hybridization. PCRex and PCRin probes were obtained by PCR amplification of a cloned spliced ad tripartite leader sequence derived from cDNA, or from plasmid containing sequences corresponding to the first intron of the MLTU, respectively. The sequences of the primers used for amplification and the oligonucleotides used as probes are given in Materials and Methods. Processed and unprocessed forms of a viral late RNA are represented by black lines. Hexon refers to the message body coding for the viral late capsid protein hexon. 1, 2, and 3 show the location of the tripartite leader exons. Gray boxes show the parts of the message complementary to the probes. The figure is not drawn to scale.

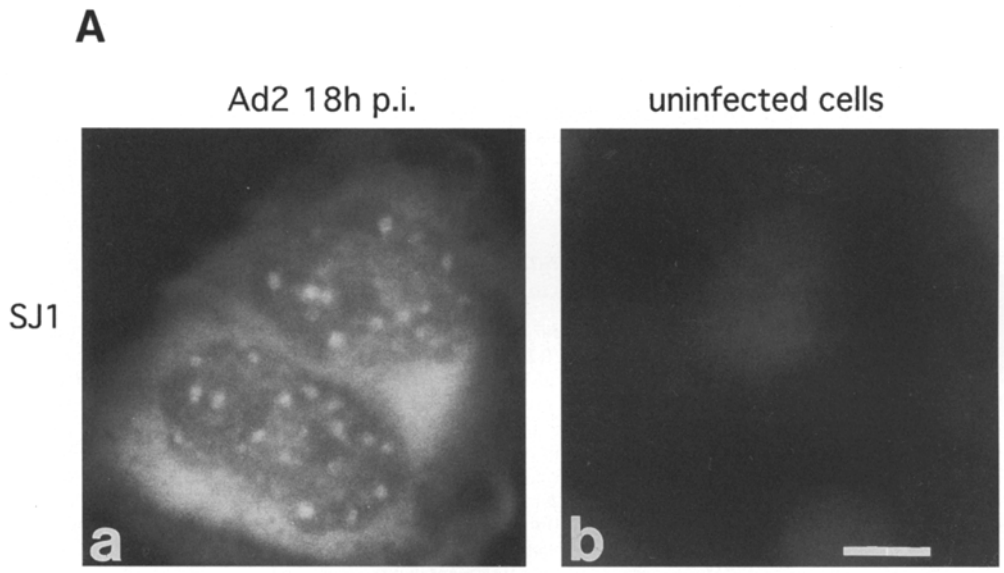


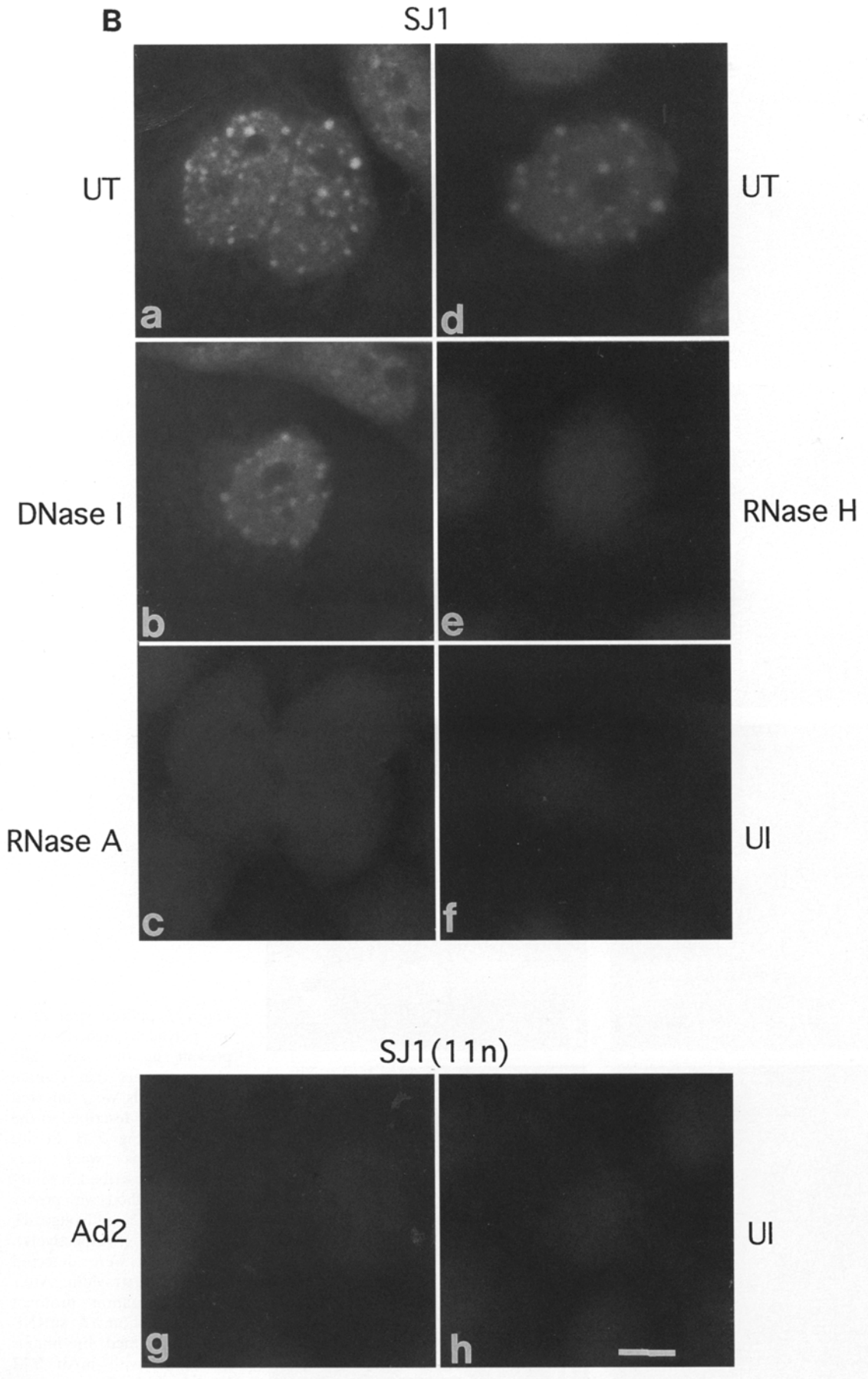
Figure 2. (*A*) In situ hybridization to viral nucleic acids. Infected cells at 18 h p.i. (*a*) and uninfected cells (*b*) grown on coverslips were fixed in paraformaldehyde and extracted in SDS buffer as described in Materials and Methods. In situ hybridizations were performed with the biotinylated oligonucleotide probe SJ1. The probe was detected with FITC-extravidin. In infected cells labeling is observed in the cytoplasm and in the nucleus. In the nucleus the staining is concentrated in several discrete spots. (*B*) SJ1 detects spliced viral RNA. Infected (*a* through *e*, and *g*) and uninfected (*UI*) (*f* and *h*) cells grown on coverslips were extracted with 0.5% Triton X-100 buffer and then fixed in paraformaldehyde as described in Materials and Methods. *a–f* show cells that were hybridized to SJ1. *g* and *h* were hybridized to SJ1(11b), which contains only the last 11n of SJ1 complementary to the 3′ end of exon 1. *UT*, untreated cells. *e* shows cells that were treated with 75 U/ml RNAse H after hybridization. *b* and *c* show cells treated with 100 U/ml DNase I and 100 μg/ml RNAse A, respectively, before hybridization. Bars: (*A* and *B*) 10 μm.

phase-infected HeLa cells using in situ hybridization. Oligonucleotide probes labeled with digoxigenin or biotin were hybridized to fixed cells that had been grown and infected on coverslips. The location of the probes used in this study relative to their target RNA sequences is shown in Fig. 1. SJ1 is an oligo of 21 nucleotides (n) complementary to sequences spanning the first splice junction in the tripartite leader. The entire target sequence of 21n is only colinear in RNAs that have spliced these two exons together. Fig. 2 *A* shows that SJ1 hybridizes extensively to RNA present in the cytoplasm of infected cells (*a*) as would be expected since its target sequence is present in all spliced late RNAs derived from the MLTU. However, there is also considerable hybridization of SJ1 to sequences in the nucleus, where the probe is concentrated in a number of discrete centers. No signal was observed with the probe in uninfected cells (*b*).

We next investigated the nature of the target sequences detected by probe SJ1 in these nuclear centers by treating fixed cells with various nucleases either before or after performing the in situ hybridization. Fig. 2 *B* shows strong hybridization of SJ1 to nuclear centers in untreated infected cells. In cells treated with RNase A before hybridization or RNase H after hybridization, no staining was observed with this probe. In contrast, infected cells treated with DNase I before hybridization showed strong labeling of the nuclear centers. In these experiments cells were preextracted with buffer containing Triton X-100 as described in Materials and Methods to remove the cytoplasmic RNA and increase the visibility of the nuclear signal. We also tested a probe that contained the last 11n of SJ1 complementary to the 3′ end of exon 1 (Fig. 1, SJ1(11b)). This probe did not hybridize to infected cells under identical conditions to those used for hybridization with SJ1 suggesting that SJ1 cannot efficiently hybridize to unspliced RNA which has a target sequence of only 11n complementary to SJ1. Other investigators have also found that splice junction oligonucleotide probes 24n in length hybridize specifically to spliced RNA (Zhang et al., 1994). Taken together these results show that SJ1 is hybridizing to spliced RNA present in discrete nuclear centers.

Spliced Viral RNAs and Polyadenylated RNAs Are Present in Late Phase snRNP Clusters

We have previously described the accumulation of splicing factors in discrete nuclear clusters during the late phase of viral infection (Bridge et al., 1993, 1995). In situ hybridizations were followed by immunostainings with monoclonal antibody (mAb) Y12 to determine the relationship between the nuclear centers containing RNA and late phase clusters containing snRNP. The results are shown in Fig. 3. SJ1 was present in nuclear centers that are identical to the late phase snRNP clusters detected by Y12 (compare *a* and *b*). Two other probes complementary to the second and third splice junctions of the viral mRNA encoding the hexon protein were also detected in the same late phase clusters that contain snRNP (*c–f*). Thus, late viral RNAs which are at least partially spliced accumulate in this structure during the late phase. The location of polyadenylated RNA in infected cells was determined with an oligo dT probe of 21n. This probe also labeled the same late phase clusters that contain snRNP (compare *g* and *h*). Oligo dT probes have been previously shown to label the splicing factor rich interchromatin granule network in uninfected cells (Carter et al., 1991, 1993; Visa et al., 1993, see also Fig. 4 *h*). Unspliced RNA and intron sequences were detected with probes USJ5, USJ6, and IN1. These probes labeled small punctate spots throughout the nucleoplasm (Fig. 4); they did not label large centers such as those detected by oligo dT (Fig. 4 *g*) that correspond to late phase snRNP clusters (Fig. 3).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SJ1
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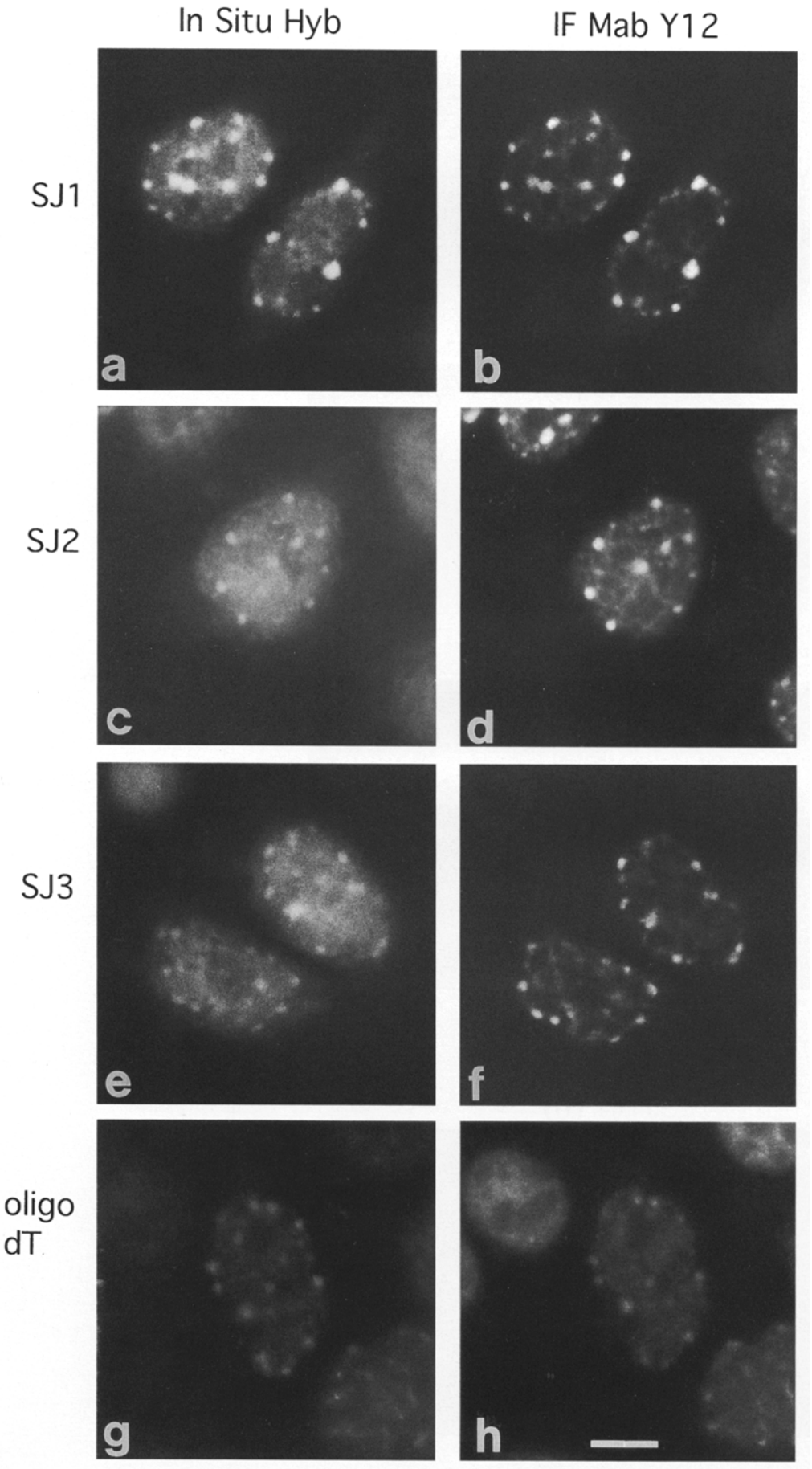

Figure 3. Spliced viral RNA and polyadenylated RNA are present in the same late phase clusters that contain snRNP. Cells were infected and fixed as described in the legend for Fig. 2 *B*. In situ hybridizations were performed as described in Materials and Methods with probes SJ1, SJ2, SJ3, and oligo dT (*a*, *c*, *e*, and *g*, respectively). The probes were detected with FITC-extravidin. After the hybridization protocol the localization of snRNP was determined by immunostaining with mAb Y12 (*b*, *d*, *f*, and *h*). Bar, 10 μm.

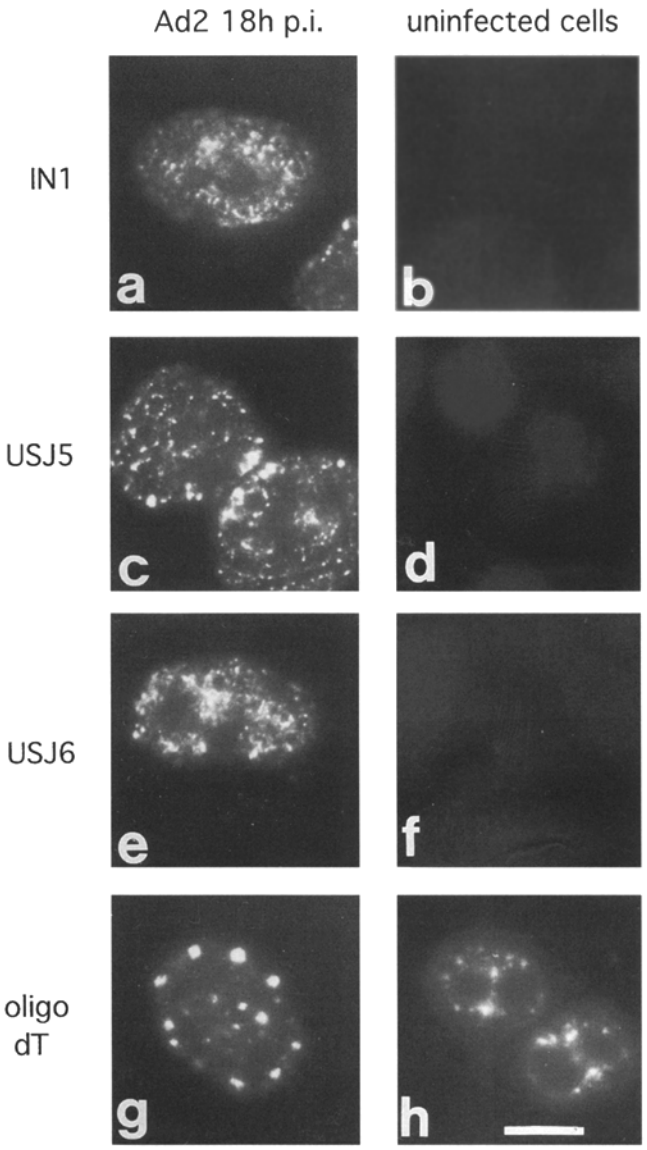

Figure 4. Localization of probes complementary to introns and unspliced junctions. Infected and uninfected cells were fixed and hybridized as described in the legend for Fig. 2 *B*. Probes IN1 (*a* and *b*), USJ5 (*c* and *d*), and USJ6 (*e* and *f*) were used for in situ hybridization and detected with anti-digoxigenin. The results are compared with the oligo dT probe (*g* and *h*) which labels snRNP clusters in infected cells (Fig. 3) and the snRNP containing interchromatin granule network in uninfected cells (*h*, Carter et al., 1991, 1993). Oligo dT was detected with FITC-extravidin. The large clusters detected with oligo dT are not observed in cells hybridized to intron or unspliced junction probes. Bar, 10 μm.

In situ hybridizations were also performed with DNA probes generated by PCR amplification of ad DNA clones in the presence of biotinylated dUTP (see Fig. 1). Probe PCRex corresponds to the 201n spliced tripartite leader exons and was amplified from a cDNA clone containing the spliced tripartite leader. Probe PCRin corresponds to 250n within the first intron of the MLTU. Late phase-infected cells were processed for in situ hybridization with these probes, and then immunostained with mAb Y12 to determine the location of the snRNP clusters. Confocal micrographs of the staining patterns are shown in Fig. 5. PCRex labels the same late phase clusters that contain snRNP; the confocal overlay of these two staining patterns shows that the clusters are yellow (Fig. 5 *c*). PCRex shows a hybridization pattern that is similar to that obtained with the splice junction oligos (Figs. 2 and 3). Thus, all of the probes we have tested that hybridize to ad tripartite leader exons label the late phase snRNP clusters. In contrast, PCRin shows staining in the nucleus that is not concentrated in snRNP clusters; the confocal overlay shows that the red snRNP clusters are separate from the green staining obtained with PCRin (Fig. 5 *f*). We have no evidence that introns or unspliced RNA accumulate in the snRNP clusters, although it should be noted that we cannot exclude the presence of unprocessed RNA in the clusters since it is always possible that the target sequences are not accessible to the probes there. Taken together, the data show that the RNA present in the late phase snRNP clusters consists of species that are at least partially spliced and polyadenylated.

Nuclear Accumulation of Spliced Viral Late RNA

The results described above indicate that there is a significant accumulation of spliced RNA within the nucleus of late phase-infected cells. We have measured the levels of spliced and unspliced leader sequences at 18–20 h p.i. using S1 nuclease analysis, and find that 85–90% of nuclear tripartite leader sequences are spliced (Fig. 6 *A*). Spliced tripartite leader sequences in the nucleus represent ~25–40% of the total spliced tripartite leader present in the cell at 18 h p.i. (data not shown) and are therefore a significant population of spliced viral late RNA. In contrast, cellular and viral early mRNAs are strongly biased towards the cytoplasmic compartment (Leppard and Shenk, 1989). To determine whether nuclear accumulation of spliced tripartite leader containing RNAs is characteristic for the biogenesis of MLTU derived transcripts or whether it results from overloading the machinery for the production of these RNAs, we performed time course experiments in which we compared the nuclear and cytoplasmic levels of spliced tripartite leader sequences. The results of this time course are shown in Fig. 6 *B*. From 11–18 h p.i., the percent of spliced leader RNA in the nucleus was ~25–35% of total spliced sequences detected in the culture. Thus, the proportion of spliced nuclear RNA to spliced cytoplasmic RNA at 11 h p.i. was not markedly different from that at 18 h p.i. At 8 h p.i., the earliest time at which we could detect spliced tripartite leader sequences, the amount of nuclear spliced RNA was actually greater than the corresponding cytoplasmic levels. These results suggest that nuclear accumulation of spliced tripartite leader RNA does not occur simply as a result of overloading the system with MLTU derived transcripts.

Tripartite Leader Sequences Are Efficiently Exported from the Nucleus

The presence of a large fraction of spliced viral tripartite leader containing RNA in the nucleus, and detection of at least a portion of these RNAs in late phase snRNP clusters, raises the possibility that the RNA present in the clusters is targeted for export to the cytoplasm. We have examined export of sequences present in viral RNAs in kinetic

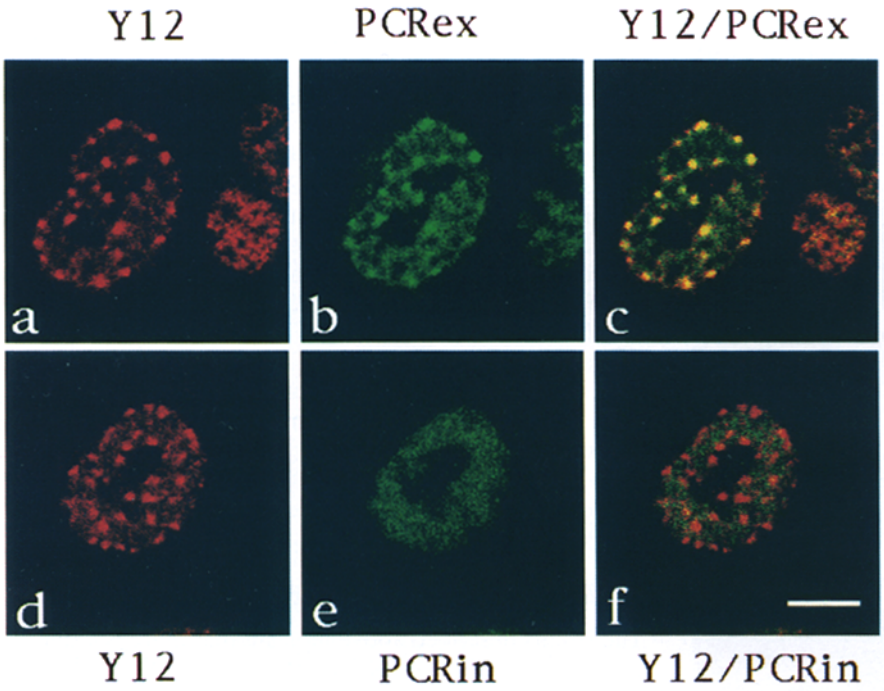

Figure 5. Confocal micrographs showing the localization of viral RNA relative to late phase snRNP clusters. Cells were infected and fixed as described in the legend for Fig. 2 *B*. Immunostaining with mAb Y12 is shown in red in *a* and *d*. Y12 was detected with a Texas red coupled secondary antibody. In situ hybridizations with probes PCRex and PCRin are shown in green in panels *b* and *e*, respectively, and were detected with FITC-extravidin. Confocal overlays of the staining patterns are shown in *c* and *f*. SnRNP clusters (*a*) and the centers labeled by PCRex (*b*) colocalize in the overlay of these two staining patterns in which the clusters are yellow (c). The snRNP clusters (*d*) are separate from the in situ hybridization staining pattern observed with PCRin (*e*); the overlay shows separate red and green staining (*f*). Bar, 10 μm.

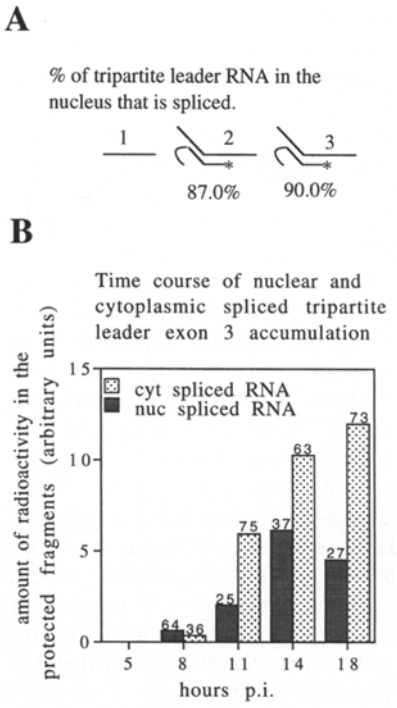

Figure 6. (*A*) S1 nuclease analysis of nuclear tripartite leader accumulation. ^{32}P-labeled oligonucleotide probes corresponding to the region overlapping the 5' end of exon 2 and the 5' end of exon 3 were protected by nuclear RNA prepared at 18 h p.i. as described in Materials and Methods. 1, 2, and 3 refer to the ad tripartite leader exons. The location of the probes used for S1 analysis is shown below exons 2 and 3. Probes were labeled at the 5' end as indicated by a star (*). The 3′ end of each probe contained additional sequences from the bacterial chloramphenicol gene so that the fragment protected by unspliced RNA could be differentiated from the full-length probe. The protected bands from spliced and unspliced nuclear infected cell RNA were quantitated by phosphorimaging. The numbers below each probe indicate the % of the radioactivity in the fragment protected by spliced RNA relative to the total radioactivity protected by both spliced and unspliced forms of nuclear RNA. The results indicated that most of the steady state RNA in the nucleus is spliced at the tripartite leader exons. (*B*) Time course analysis of nuclear and cytoplasmic tripartite leader accumulation. Cells were infected with ad, and nuclear and cytoplasmic RNA was prepared as described in Materials and Methods at the indicated times. An oligonucleotide probe overlapping the 5′ end of the third exon of the tripartite leader (Fig. 6 *A*) was used to measure accumulation of this RNA by S1 analysis. The amount of labeled probe protected by spliced nuclear and spliced cytoplasmic RNA at different times after infection was quantitated by phosphorimaging. Numbers above the columns give the percentage of spliced RNA in nuclear and cytoplasmic compartments for each time point.

labeling experiments of cells incubated with [^{3}H]uridine from 18 h p.i. After a 2-h continuous label with [^{3}H]uridine, uninfected cells and cells from 20 h p.i. show no difference in the total amount of labeled polyadenylated RNA that has been exported to the cytoplasm during the labeling period (data not shown, see also Flint, 1986). Thus, there is no defect in the transport of mRNA from the nucleus to the cytoplasm during this phase of the infection even though significant levels of spliced RNAs have accumulated in the nucleus.

We have looked at the conservation of newly synthesized RNA between the nucleus and the cytoplasm by following the rate of [^{3}H]uridine incorporation into a target sequence in both the nucleus and the cytoplasm during continuous labeling (Nevins and Darnell, 1978). If newly synthesized sequences are conserved between the nucleus and the cytoplasm then the rate of incorporation of label into the nuclear target sequence during the initial linear phase of the labeling period, will be the same as the rate of

accumulation of label into the cytoplasmic target sequence. Thus, the slope of the curves showing the amount of radioactivity incorporated into the target sequence will be similar in both the nucleus and the cytoplasm, but the curve showing the incorporation into cytoplasmic RNA will be delayed relative to the nuclear curve due to the time it takes for the target RNA to be exported to the cytoplasm. Previous studies using these techniques were consistent with the view that every transcript produced from the MLTU is processed to one mRNA which is exported to the cytoplasm (Nevins and Darnell, 1978). We compared the conservation between the nucleus and the cytoplasm of spliced tripartite leader sequences and sequences corresponding to late region 3 (L3) in the experiments shown in Fig. 7. We find that the slope of the curves showing incorporation of label into tripartite leader containing nuclear and cytoplasmic RNA differed by less than twofold suggesting that the newly synthesized tripartite leader is efficiently exported to the cytoplasm (Fig. 7 *A*). In contrast the slope of the nuclear accumulation curve of newly synthesized L3 sequences was about six times greater than the corresponding slope of the accumulation curve in the cytoplasm. This shows that only ~15% of newly synthesized L3 is exported to the cytoplasm as would be expected since this region is processed away from mRNAs produced from each of the other four late regions (Nevins and Darnell, 1978; Sharp, 1984). The results obtained with the tripartite leader sequences are in good agreement with the results showing 70–100% conservation of sequences at the 3′ end of poly(A) selected viral RNA between the nucleus and the cytoplasm (Nevins and Darnell, 1978); the rate of tripartite leader containing RNA accumulation in the cytoplasm was within twofold of the nuclear value. These results indicate that viral spliced tripartite leader sequences are being transported between the nucleus and the cytoplasm about as efficiently as viral polyadenylated RNA during the late phase of infection.

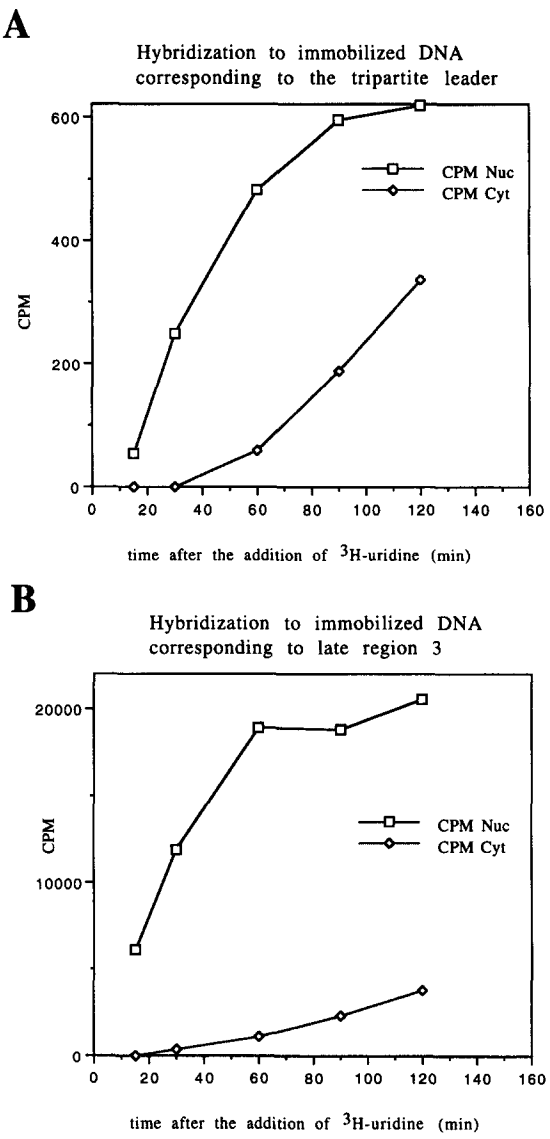

Figure 7. [^{3}H]Uridine incorporation into nuclear and cytoplasmic viral RNA. Cells at 18 h p.i. were incubated with media containing 0.2mCi/ml [^{3}H]uridine and cold uridine to a final concentration 14 μM uridine. At the times indicated the labeled media was removed and RNA was prepared from the cytoplasmic and nuclear fractions as described in Materials and Methods. Cytoplasmic or nuclear RNA from approximately 6×10^6 cells was hybridized to filters of immobilized plasmid DNA containing the 201n spliced tripartite leader sequence derived from a cDNA clone (*A*) or to immobilized plasmid DNA containing sequences from late region 3 (*B*). pGem11 vector DNA without any viral insert was used as a control to determine the nonspecific binding. The amount of radioactivity hybridizing to these filters was determined by scintillation counting. The values are given in counts per minute (cpm) and have been corrected for the background hybridization to pGem 11 DNA.

Discussion

The biochemical events in eukaryotic RNA production have been extensively investigated; less is known about the nuclear organization of gene expression. Eukaryotic gene expression is characterized by separate cellular compartments for the synthesis of RNA and the translation of proteins from that RNA. Thus, RNAs that encode for proteins must move from the site of their transcription within the nucleus to the nuclear pore where they are transferred to the cytoplasm; this process has been described as RNA export (for reviews see Izaurralde and Mattaj, 1995; Gerace, 1995). The mechanisms and the factors involved in regulating RNA export are only beginning to be defined, and the manner in which the export of RNA is coordinated with posttranscriptional processing events such as polyadenylation and splicing is not well understood. Determining the nuclear localization of RNA will be an important aspect of understanding how RNA production is organized within the nuclear compartment. We have used in situ hybridization techniques to localize ad RNA in late phase-infected cells.

An oligonucleotide probe complementary to the first splice junction of the ad tripartite leader (Fig. 1), hybridized to sequences present in the cytoplasm of infected cells as would be expected, but in addition, we found that this probe hybridized to RNA present in discrete centers in the nucleus (Fig. 2). Double-labeling experiments in which the in situ hybridization was followed by immunostaining,

showed that the nuclear centers that are labeled by the splice junction and exon probes are identical to the snRNP clusters which we have previously described in ad-infected cells (Bridge et al., 1993, 1995; Fig. 3 and Fig. 5, *a–c*). We have provided evidence that snRNP clusters are likely to correspond to enlarged interchromatin granules present in late phase-infected cells (Bridge et al., 1993). This is confirmed by electron microscopy studies showing that late during infection enlarged interchromatin granules contain high concentrations of snRNP in addition to viral RNA and polyadenylated RNA (Puvion-Dutilleul et al., 1994). Polyadenylated RNA is also present in the interchromatin granule network in uninfected HeLa cells (Carter et al., 1991, 1993; Visa et al., 1993; see also Fig. 4 *h*). We and others have previously detected ad RNA at the site of transcription in intermediate phase cells (Pombo et al., 1994) and in cells limited to the early phase of gene expression (Zhang et al., 1994). Here we show that in late phase ad-infected cells, the interchromatin granule clusters that contain splicing factors also accumulate spliced RNA from the MLTU. Thus, RNA that has undergone posttranscriptional processing events of polyadenylation and/or splicing accumulates in a specific nuclear structure during the late phase of viral gene expression.

Although interchromatin granules are known to contain RNA in both infected and uninfected cells, the available data suggests that this structure is not a location for viral or cellular transcription (Pombo et al., 1994; Puvion-Dutilleul and Puvion, 1991; Fakan, 1994). Rather, RNA accumulates in the interchromatin granules posttranscriptionally. During infection by ad, considerable levels of both processed and unprocessed forms of viral RNAs accumulate in the nucleus during the late phase (Berget and Sharp, 1979; Manley et al., 1979). We show here that ~85% of the tripartite leader RNA present in the nucleus is spliced, and this represents about one third of the total spliced tripartite leader present in the cell (Fig. 6 *A* and data not shown). This accumulation of spliced RNA in the nucleus is observed even at the onset of the late phase (Fig. 6 *B*). Thus, the nuclear accumulation of spliced RNA appears to be a characteristic of viral late RNA biogenesis. Leppard and Shenk (1989) examined ad RNA metabolism using a nuclear fractionation scheme to operationally define several nuclear compartments. Viral early RNA and cellular RNAs were strongly biased towards the cytoplasmic compartment; very little RNA corresponding to the viral early E1a transcript or the cellular β-actin gene was found in any nuclear subfraction although the levels of these RNAs in the cytoplasm were substantial. In contrast, MLTU derived RNAs showed significant accumulation in several nuclear subfractions. Pulse-chase analyses also showed that late RNAs accumulate in specific nuclear subfractions before their export to the cytoplasm. In particular, pulse-labeled late RNAs accumulated most extensively in a nuclear compartment designated F3; this compartment was not transcriptionally active. Movement through the F3 compartment was suggested as a possible rate-limiting step in the production of viral RNA (Leppard and Shenk, 1989). Thus, our demonstration that processed MLTU derived RNA accumulates in the interchromatin granule structure is consistent with previous biochemical investigations showing that viral late RNA proceeds through particular nuclear subfractions before its export to the cytoplasm. The relationship between the late RNA present in the biochemically defined F3 nuclear subfraction and the late RNA present in the interchromatin granule clusters remains to be investigated, but the possibility that they represent the same RNA subpopulation is intriguing.

It is well established that only a fraction of the MLTU derived sequences synthesized in the nucleus are transported to the cytoplasm (Nevins and Darnell, 1978; see also Fig. 7 *B*). In contrast, the sequences close to the viral poly(A) sites in newly synthesized polyadenylated RNA from the MLTU were conserved between the nucleus and cytoplasm (Nevins and Darnell, 1978). These experiments suggested that each primary transcript from the MLTU was polyadenylated and spliced to give rise to one mRNA that was subsequently exported to the cytoplasm. The RNA processed away from this transcript would be degraded in the nucleus and never appear in the cytoplasm. This model accounts for the observation that only ~14% of the newly synthesized MLTU RNA is exported to the cytoplasmic compartment (Nevins and Darnell, 1978). Furthermore, the model predicts that the tripartite leader exons should be efficiently exported since they are present on the 5′ ends of each MLTU derived mRNA. We observed that spliced tripartite leader sequences were present in the nuclear interchromatin granule structures (snRNP clusters) (Fig. 3 and Fig. 5, *a–c*). To confirm that the tripartite leader is efficiently transported from the nucleus to the cytoplasm at a time when we see significant accumulation of tripartite leader RNA in the snRNP clusters, we have performed kinetic labeling experiments similar to those described by Nevins and Darnell (1978) at 18 h p.i. Our data (Fig. 7) suggest that RNA sequences hybridizing to spliced tripartite leader containing DNA are about as well conserved as sequences at the 3′ end of poly(A) selected viral RNAs (Nevins and Darnell, 1978), and confirm the prediction that newly synthesized tripartite leader RNA is efficiently transported from the nucleus to the cytoplasmic compartment during the viral late phase.

What is the significance of the interchromatin granule compartment in the production of viral RNA? Since mRNAs that are targeted to the cytoplasm are spliced and polyadenylated, the presence of spliced RNA from the ad tripartite leader as well as polyadenylated RNA raises the possibility that viral MLTU RNAs accumulate in the interchromatin granule structure during the process of RNA transport. It is difficult to rule out the alternative possibility that the tripartite leader RNA present in the interchromatin granule represents RNAs that are targeted for nuclear degradation rather than export to the cytoplasm. However, the relative conservation of the tripartite leader exons between the nucleus and the cytoplasm argues against this interpretation. Huang et al. (1994) find that polyadenylated RNA remains stably associated with the interchromatin granule following treatment with transcription inhibitors; they suggest that this is not mRNA but rather represents a population of stable RNA involved in other nuclear functions. In contrast, Visa et al. (1993) find that levels of polyadenylated RNA in interchromatin granules are significantly decreased after treatment with actinomycin D. Thus, it is not yet clear if the RNA in the interchromatin granule is stable or if there is continual flux

of RNA into and out of this compartment. Our data does not exclude the possibility that some of the polyadenylated RNA detected by in situ hybridization is stably associated with the interchromatin granule, perhaps serving a structural function. However, the nuclear localization of tripartite leader splice junctions and exons in the same late phase clusters that contain snRNP (Fig. 3 and Fig. 5, *a–c*) shows that sequences present in the 5′ end of spliced adenovirus mRNAs are also located in the interchromatin granule. Several other cellular and viral RNAs have been detected in the interchromatin granule; all of these RNA sequences are also detected in the cytoplasmic compartment (Besse et al., 1995, 1996; Besse and Puvion-Dutilleul, 1996; Xing et al., 1995). Xing et al. (1995) have found a close association between several transcribing cellular RNAs and the interchromatin granule. In particular, tracks of collagen Iα1 RNA were found to extend into the interchromatin granule from the transcribing gene located at the periphery. The majority of the collagen Iα1 RNA detected in this structure appeared to have spliced out at least one intron. Further evidence for the role of the interchromatin granule in the accumulation of spliced RNA comes from the characterization of three antibodies directed against nuclear matrix antigens (Blencowe et al., 1994). These antibodies were found to label the splicing factor containing interchromatin granules in situ. Interestingly, all three of the nuclear matrix antibodies preferentially immunoprecipitated splicing complexes that contained exon sequences. These data are all consistent with a model in which the interchromatin granule accumulates processed RNA posttranscriptionally; we suggest that this RNA may be in transit from the site of transcription to the cytoplasmic compartment.

Ad infection prevents newly synthesized cellular RNA from appearing in the cytoplasm during the late stages of infection (Flint, 1986; Sarnow et al., 1984; Pilder et al., 1986; Cutt et al., 1987). Thus, the late phase provides a mechanism for the selective export of viral RNAs. It is possible that the high concentration of viral RNA in the interchromatin granule is an important feature of this process. Herpes virus RNAs also accumulate in enlarged interchromatin granule structures (Besse et al., 1995, 1996), and the late phase of herpes virus infections is associated with defective expression of cellular mRNAs (Smiley et al., 1991). The selective export of viral RNAs during the late phase of ad infection is thought to be mediated by a complex of the E1b 55K and E4 ORF 6 34K proteins (Flint, 1986; Pilder et al., 1986; Sarnow et al., 1984; Cutt et al., 1987). Further studies addressing the role of these proteins in the nuclear trafficking of viral RNA may begin to address the mechanisms through which viral RNAs are selectively exported during the late phase of infection.

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