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Localization of HIV-1 RNA in Mammalian Nuclei

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Abstract. The Rev protein of human immunodeficiency virus type 1 (HIV-1) facilitates the nuclear export of unspliced and partially spliced viral RNAs. In the absence of Rev, these intron-containing HIV-1 RNAs are retained in the nucleus. The basis for nuclear retention is unclear and is an important aspect of Rev regulation. Here we use in situ hybridization and digital imaging microscopy to examine the intranuclear distributions of intron-containing HIV RNAs and to determine their spatial relationships to intranuclear structures. HeLa cells were transfected with an HIV-1 expression vector, and viral transcripts were localized using oligonucleotide probes specific for the unspliced or spliced forms of a particular viral RNA. In the absence of Rev, the unspliced viral RNAs were predominantly nuclear and had two distinct distributions. First, a population of viral transcripts was distributed as ~10–

20 intranuclear punctate signals. Actinomycin D chase experiments indicate that these signals represent nascent transcripts. A second, stable population of viral transcripts was dispersed throughout the nucleoplasm excluding nucleoli. Rev promoted the export of this stable population of viral RNAs to the cytoplasm in a time-dependent fashion. Significantly, the distributions of neither the nascent nor the stable populations of viral RNAs coincided with intranuclear speckles in which splicing factors are enriched. Using splice-junction-specific probes, splicing of human β -globin pre-mRNA occurred cotranscriptionally, whereas splicing of HIV-1 pre-mRNA did not. Taken together, our results indicate that the nucleolus and intranuclear speckles are not involved in Rev regulation, and provide further evidence that efficient splicing signals are critical for cotranscriptional splicing.

THE human immunodeficiency virus type 1 (HIV-1)¹ Rev protein acts posttranscriptionally to selectively increase the cytoplasmic levels of the *gag-pol* and *env* mRNAs (Dayton et al., 1986; Feinberg et al., 1986; Rosen et al., 1988; Emerman et al., 1989; Felber et al., 1989; Malim et al., 1989a). A *cis*-acting RNA element (Rev response element [RRE]) that is required for Rev function has been mapped within the *gag-pol* and *env* mRNAs (Hammariskjold et al., 1989; Malim et al., 1989a; Hadzopoulou-Cladaras et al., 1989; Huang et al., 1991; Green, 1993).

Rev contains an RNA-binding domain, required for interaction with HIV-1 RNA, and an effector domain, required for RNA-bound Rev to function (Daly et al., 1989; Dayton et al., 1989; Zapp and Green, 1989; Heaphy et al., 1990; Malim et al., 1990; Olsen et al., 1990; Bartel et al., 1991; Kjems et al., 1991a). It is thought that the Rev effector domain interacts with a cellular cofactor required to mediate the Rev response (Vaishnav et al., 1991; Ruhl et al., 1993; Luo et al., 1994; Bogerd et al., 1995; Fritz et al.,

1995). Recently, hRIP (also called Rab), a human nucleoporin-like protein that specifically interacts with the Rev effector domain and has the properties expected of the Rev cellular cofactor, has been identified (Bogerd et al., 1995; Fritz et al., 1995).

Although biochemical and genetic studies have shown that, in the absence of Rev, incompletely spliced HIV-1 pre-mRNAs are retained in the nucleus, the intranuclear distribution of these viral RNAs has not been well defined. Based primarily upon the intranuclear distribution of Rev, two subnuclear structures have been proposed to be important for Rev function. First, Rev itself is nuclear localized and concentrated in the nucleolus (Malim et al., 1989b; Nosaka et al., 1993; Kallard et al., 1994; Meyer and Malim, 1994). Based upon this nucleolar concentration and other considerations, it has been proposed that the nucleolus plays an intimate role in Rev function. Second, in the nucleoplasm Rev has been reported to have a speckled distribution (Kallard et al., 1994). This speckled pattern coincides with that of several cellular splicing factors (for review see Spector, 1993).

While these previous studies have localized Rev, they did not analyze the viral RNAs upon which Rev acts. We have developed methods to specifically localize DNA, intronic RNA, or spliced RNA by fluorescent in situ hybridization using specific oligonucleotide probes (Zhang et al., 1994). Here, we have adapted this approach to localize

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1. *Abbreviations used in this paper:* IN, intron; HIV-1, human immunodeficiency virus type 1; RRE, Rev response element; SJ, splice-junction; SJ-C, splice-junction control.

HIV-1 RNAs and to determine their relationship to intranuclear structures.

Materials and Methods

Transient Transfection Assays

HeLa cells were grown on sterile, gelatin-coated glass coverslips to 75% confluency. Transient transfection assays for Rev function were performed using the calcium phosphate precipitation DNA transfection protocol described by Lin and Green (1991). Transient transfection reactions in the absence of Rev contained 3 µg pgTAT DNA (Malim et al., 1988); reactions in the presence of Rev contained 3 µg pgTAT and 3 µg pcREV DNAs. Total DNA volume in each transfection mix was adjusted to 20 µg

with the empty plasmid, pGEM3 (Promega, Madison, WI). Transfected HeLa cells were analyzed at 12, 24, 36, 48, and 72 h after transfection.

In Situ Hybridization

After transfection, the coverslips were incubated in fixation buffer (4% paraformaldehyde, 1× PBS [1 mM KH₂PO₄, 10 mM Na₂HPO₄, 0.13 M NaCl, 2.7 mM KCl, pH 7.0], 5 mM MgCl₂) for 15 min, and then rinsed and stored in 70% ethanol at 4°C until use. In situ hybridization reaction conditions were performed as described (Zhang et al., 1994).

Digital Imaging Microscopy

Images were captured using a cooled charged-coupled device (CCD) camera, digitalized, and analyzed as described (Taneja et al., 1992). Green and

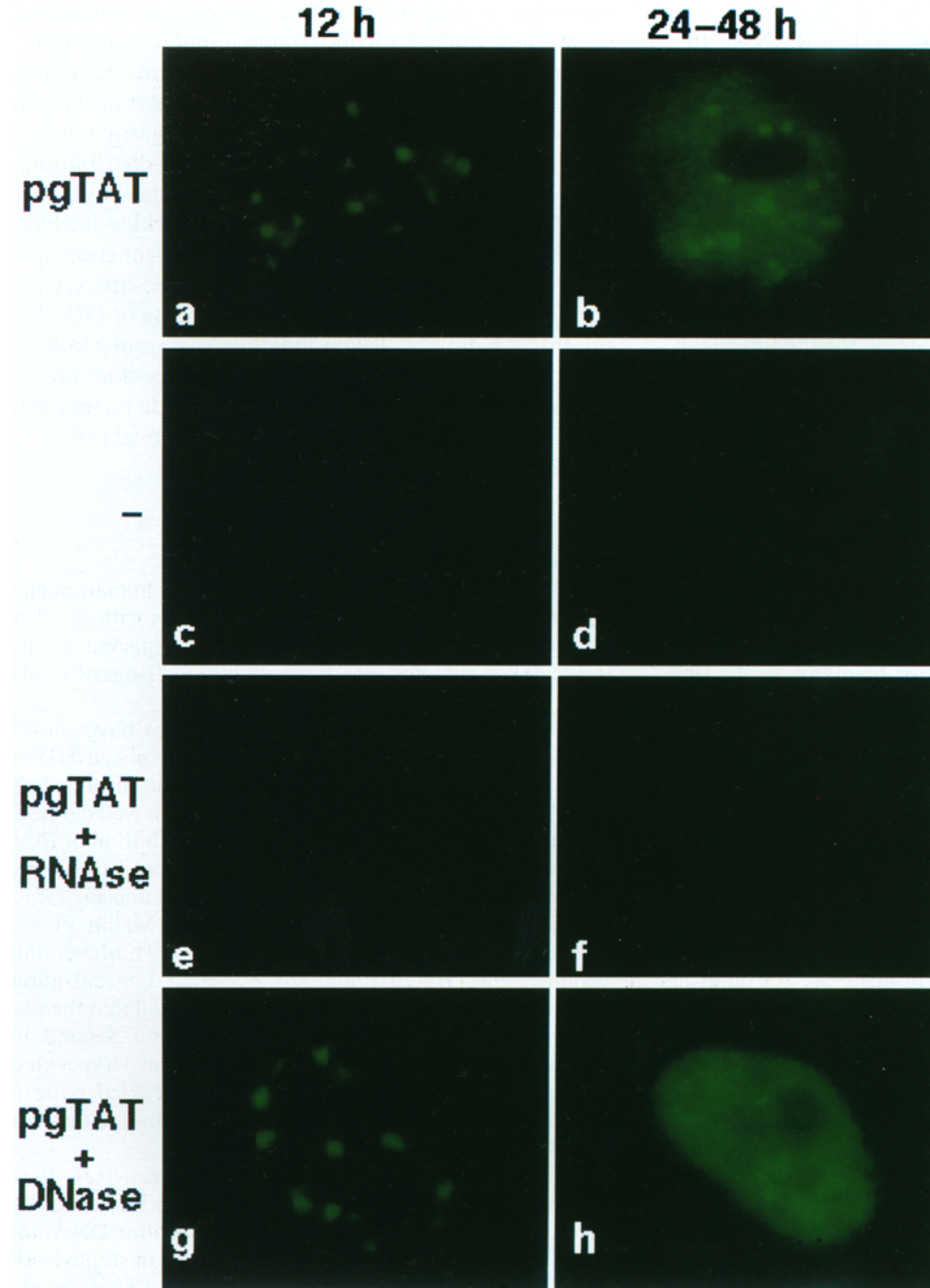


Figure 1. Intranuclear distribution of HIV-1 *tat* RNAs. HeLa cells were transfected with the pgTAT expression plasmid and incubated for the times indicated above. Cells were treated with fixative, and in situ hybridization was performed according to Zhang et al. (1994) using a *tat* intron-specific probe. *pgTAT*, pgTAT-transfected HeLa cells; (-), mock-transfected HeLa cell control; *RNase A*, pgTAT-transfected HeLa cells incubated with RNase A (100 µg/ml) before hybridization; *DNase*, pgTAT-transfected HeLa cells with RQ DNase I (5 U/30 µl) before hybridization.

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red pseudo-colored images from the same optical plane were superimposed using dual-colored fluorescence beads as fiduciary markers.

Oligonucleotide Probes

Fluorescent Labeling. Oligonucleotides containing amino-modified thymine (Glen Research Corp., Sterling, VA) were synthesized on an automated synthesizer (model 391; Applied Biosystems, Inc., Foster City, CA), purified on a 8 M urea/10% polyacrylamide denaturing gel cast in 0.5× TBE, and labeled with FITC (Molecular Probes, Inc., Eugene, OR) or CY3 (GIBCO BRL, Gaithersburg, MD) in a 100-fold excess of 0.1 M NaHCO₃/Na₂CO₃ (pH = 9.0) overnight. After this incubation, oligonucleotides were purified through a Sephadex-G50 column and resolved on a 8 M urea/10% polyacrylamide denaturing gel.

Oligonucleotide Probe Sequences. The nucleotide sequence, its location within the HIV-1 genome, and its designation is described for each oligonucleotide probe: (a) HIV-1 *tat* intron specific; IN: 5'-GTGGTTGCTTCCTTCCACACAGGT-3' and 5'-TAGGCCTGTGTAATGACTGAGGTGT-3'. Probes are complementary to nucleotides 603–626 and 1067–1090, respectively, of the *env* gene. (b) HIV-1 *tat* splice-junction specific; SJ: 5'-TGGGAGGTGGGT and TGCTTTGATAGA-3'. Probe is complementary to the splice-junction nucleotides 5614–5625/7956–7967 (/ = splice junction). (c) HIV-1 *tat* splice-junction control probe; SJC: probe contains the same two sections of splice-junction sequences, but paired in the reverse order. Probe complementary to nucleotides 7956–7967/5614–5625. (d) Human β -globin intron specific; H β -IN: 5'-TCCACATGCCAGTTTCTATTGGT-3' and 5'-TGTTATACACAATGTAAAG-

GCATT-3'. Probes are complementary to nucleotides 177–200 and 737–760 of the human β -globin gene, respectively. (e) Human β -globin splice-junction specific; H β -SJ: 5'-ACCACCAGCAGC/CTGCCAGGGCC-3' and 5'-GTTGCCAGGAG/CCTGAAGTTCTC-3'. Probes are complementary to splice-junction sequences of 131–142/273–284 and 484–495/1346–1357, respectively. (f) Human β -globin splice-junction control; H β -SJC: 5'-GCTGCTGGTGGT/GGCCCTGGGCAG-3' and 5'-CTCCTGGCAAC/TGAGAACTTCAGG-3'. Probes are complementary to the sequences of 273–284/131–142 and 1346–1357/484–495, respectively.

Results

Two Distinct Intranuclear Distributions of Intron-containing HIV-1 RNAs

To determine the intranuclear distribution of intron-containing HIV-1 RNAs, HeLa cells were transfected with a previously described HIV-1 *tat* expression vector (pgTAT; Malim et al., 1989a), and viral transcripts were localized using fluorochrome-labeled oligonucleotide probes complementary to the *env* region. Fig. 1 shows that ~10–20 punctate intranuclear dots were visualized at 12 h after transfection (Fig. 1 a). By 24–48 h after transfection, a predominantly disperse nucleoplasmic distribution was ob-

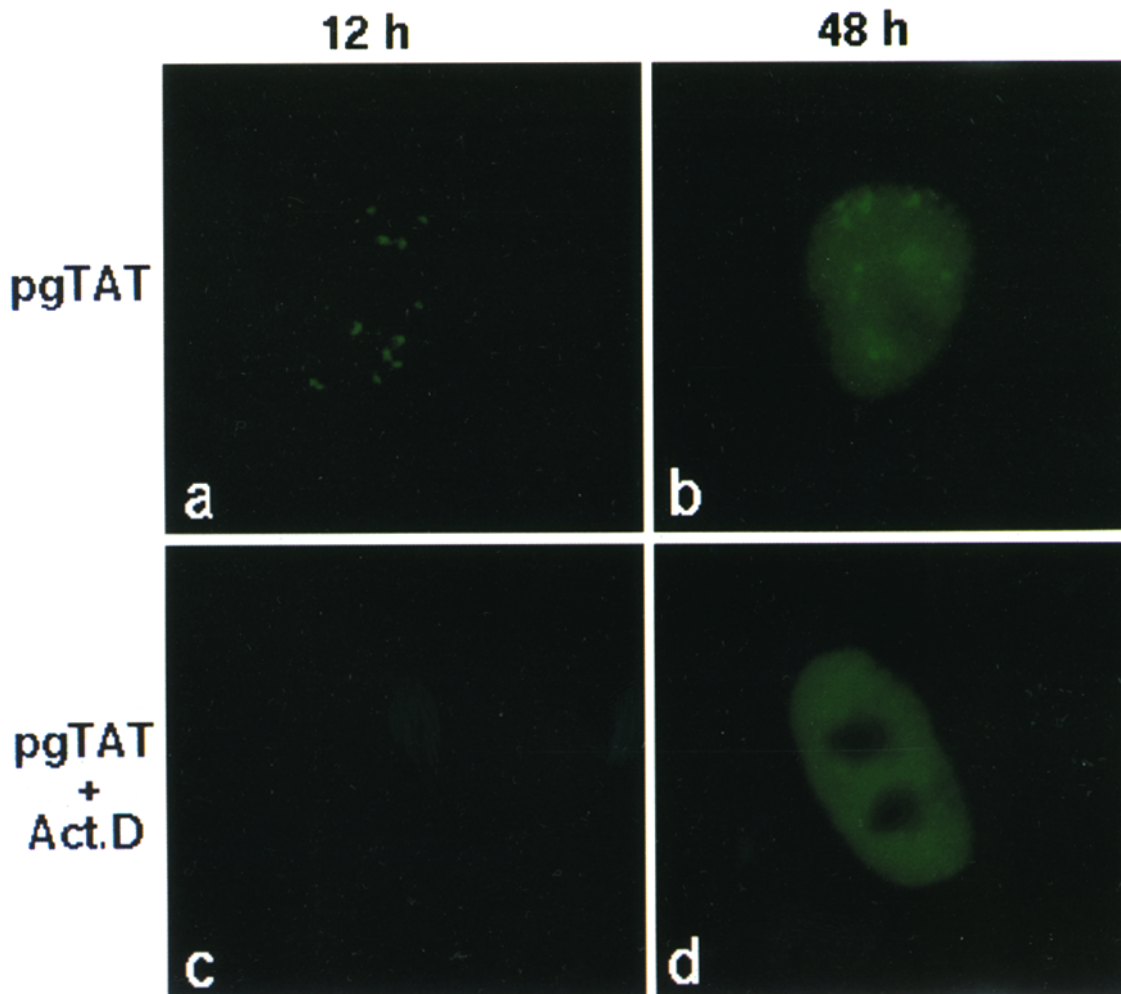


Figure 2. Actinomycin D chase experiment. The distributions of the nascent and the stable populations of HIV-1 *tat* RNA were determined using in situ hybridizations with *tat* intron-specific probes. *pgTAT*, *pgTAT*-transfected HeLa cells; *pgTAT* + Act. D, transfected HeLa cells treated with actinomycin D (10 μ g/ml) for 2 h before fixation.

served (Fig. 1 *b*; see below). At later times, the disperse nucleoplasmic pattern became so intense that it partially obscured the punctate nuclear signals (Fig. 1 *h*; data not shown). Significantly, these nuclear-localized intron-containing RNAs were completely excluded from the nucleoli.

Several control experiments confirmed that both the punctate and disperse nuclear signals were specific for HIV-1 *tat* RNAs (Fig. 1). First, nontransfected HeLa cells did not contain any signals after hybridization with the same probes (Fig. 1, *c* and *d*). Second, RNase treatment completely abolished the punctate and diffuse signals in the transfected cells (Fig. 1, *e* and *f*), whereas DNase treatment had no effect (Fig. 1, *g* and *h*). On the basis of these results, we conclude that the punctate and disperse signals are specific for HIV-1 *tat* RNAs.

To determine the relationship between these hybridization signals and transcription, cells were treated with actinomycin D (*Act. D*; Fig. 2), a specific inhibitor of RNA polymerase II. Fig. 2 shows that treatment with actinomycin D abolished the punctate (compare Fig. 2, *a* and *c*), but not the disperse (compare Fig. 2, *b* and *d*), nuclear signals. Based upon the results of Figs. 1 and 2, our previous study (Zhang et al., 1994), and data presented below, we interpret these data as follows: the punctate nuclear signals represent nascent transcripts that are released, giving rise to the predominantly disperse, stable nucleoplasmic population.

Rev Selectively Affects Nuclear Export of the Dispersed Nucleoplasmic HIV-1 RNAs

Intron-containing HIV-1 pre-mRNAs must be exported from the nucleus for expression of viral structural proteins, a process that is absolutely dependent on HIV-1 Rev. We

therefore examined the effect of Rev on both the nascent and the stable populations of HIV-1 *tat* RNAs. Cells were transfected with pgTAT DNA in the presence or absence of a Rev expression plasmid (pcREV) (Malim et al., 1989a), and the localization of *tat* RNAs was analyzed at various times after transfection (Fig. 3). In the absence of Rev, *tat* RNAs were distributed as punctate nuclear dots at early times (Fig. 3 *a*) and as a predominantly disperse distribution at 24–48 h after transfection (Fig. 3, *b–d*). Rev promoted nuclear export of the dispersely distributed viral RNAs in a time-dependent fashion (Fig. 3, *e–h*), with a maximal effect at 36–48 h after transfection (Fig. 3, *g* and *h*). Thus, as expected, the nuclear export of these viral RNAs is Rev dependent.

The specificity of Rev action in this assay was confirmed by in situ hybridization of HeLa cells transfected with a pgTAT derivative lacking an RRE (pHd/B2; Malim et al., 1989a). Fig. 4 shows that Rev did not affect the nuclear export of the unspliced pH d/B2 *tat* RNA, confirming that Rev-mediated nuclear export is specific for RRE-containing RNAs (Daly et al., 1989; Hadzopoulou-Cladaras et al., 1989; Zapp and Green, 1989; Heaphy et al., 1990; Malim et al., 1990; Olsen et al., 1990; Huang et al., 1991; Kjems et al., 1991b).

Spatial Relationship of Nucleoplasmic HIV-1 RNAs to Intranuclear Speckles

Pre-mRNA splicing factors are distributed nonrandomly in mammalian nuclei. Our recent work has demonstrated that the nucleus is not functionally compartmentalized with respect to transcription and splicing (Zhang et al., 1994). Here, we have examined the distributions of the nascent and stable populations of HIV-1 transcripts relative

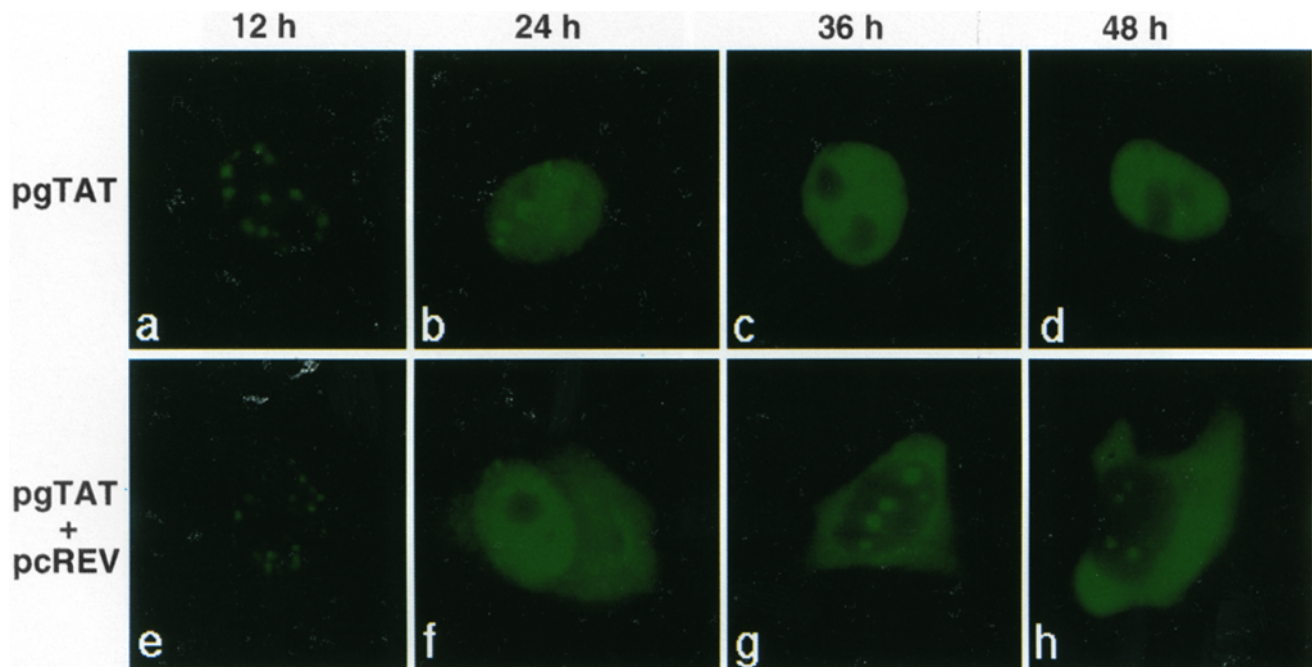


Figure 3. Effect of Rev on the localization of HIV-1 *tat* RNAs. HeLa cells were transfected with either pgTAT (*a–d*) or pgTAT and pcREV (*e–h*) expression plasmids and analyzed by in situ hybridization. Oligonucleotide probes were as described in Figs. 1 and 2 (see also Materials and Methods).

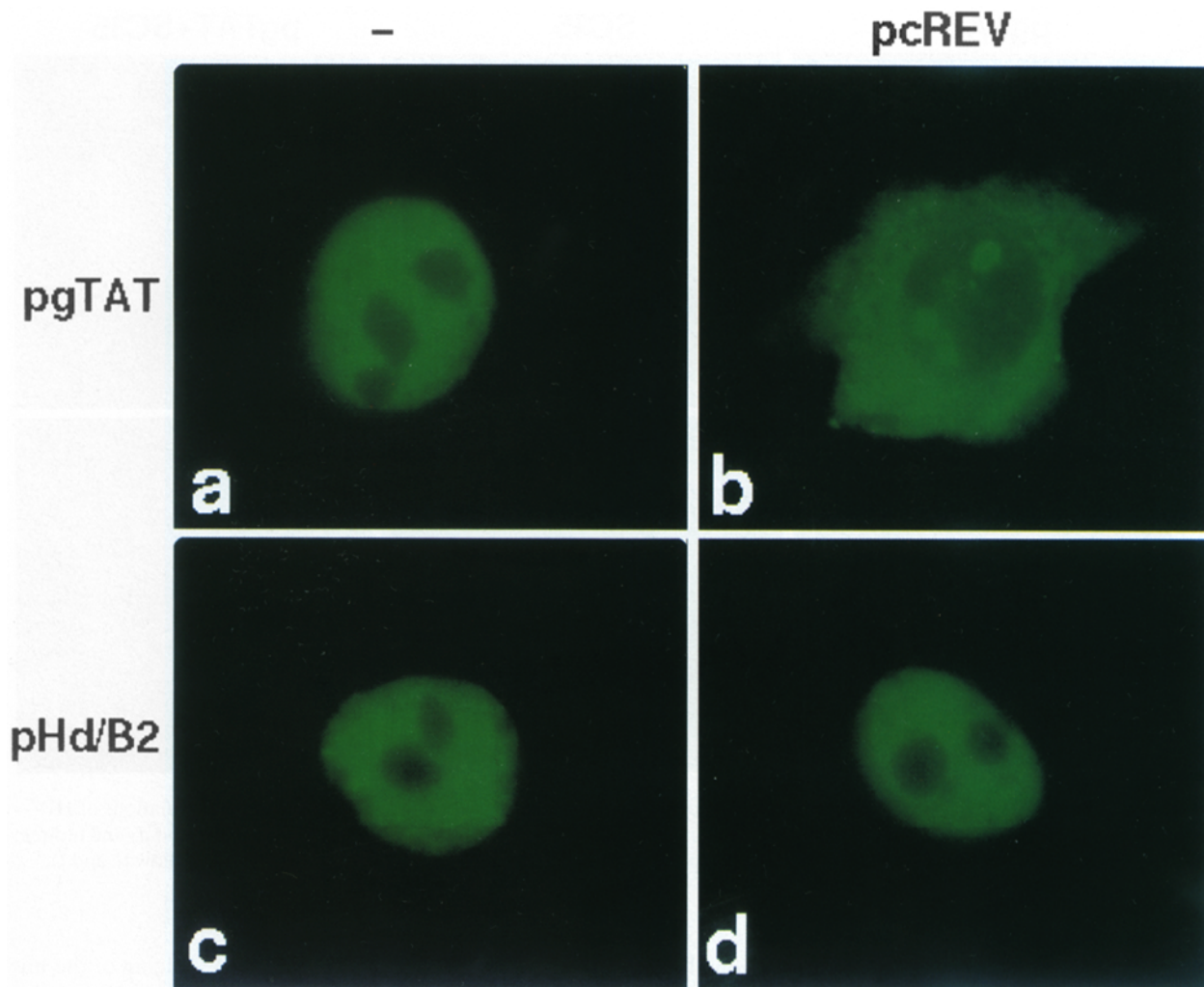


Figure 4. Rev action is dependent upon the RRE. HeLa cells were transfected with the pgTAT expression plasmid derivative, pHd/B2, in the presence (d) or absence (c) of pcREV. The nuclear distributions of wild-type *tat* intron RNAs in the presence (b) or absence (a) of Rev are indicated.

to intranuclear speckles in which splicing factors are concentrated. Once again, the viral RNA sites were determined using an intron-specific probe, and these same cells were stained with an antibody that recognizes the essential splicing factor SC35, a well-established marker for speckles (Fu and Maniatis, 1990; Spector, 1993). Fig. 5 shows that the SC35 antigen had the expected punctate distribution (Fig. 5, b and c). However, the overlay (Fig. 5, c and f) of the signals from in situ hybridization (Fig. 5, a and d) and indirect immunofluorescence (Fig. 5, b and e) indicates that these distributions did not colocalize. (Overlap of the red and green signals results in yellow.) Thus, the distributions of neither the nascent (Fig. 5, a-c) nor the stable (Fig. 5, d-f) populations of Tat transcripts coincided with that of the intranuclear speckles. These results further demonstrate that transcription and splicing are not restricted to these intranuclear compartments.

Nucleoplasmic Distribution of Spliced HIV-1 RNAs

An important aspect of Rev regulation is that the intron-

containing HIV-1 RNAs upon which Rev acts lack efficient splicing signals (Chang and Sharp, 1989). To understand the biogenesis of HIV-1 pre-mRNA in greater detail, we have determined the spatial relationship of unspliced and spliced HIV-1 *tat* RNAs by double-label in situ hybridization using intron (IN) and splice-junction (SJ) probes (IN and SJ; Fig. 6). Whereas the intron-containing RNAs were readily visualized (Fig. 6, a, d, and g), between 12 and 24 h after transfection, spliced RNA was not detected in either the nucleus or cytoplasm (Fig. 6, b and e). By 48 h, the accumulated spliced RNA in the cytoplasm was detected as a relatively disperse distribution, but again the splice-junction probe failed to detect nuclear signals.

The specificity of the HIV-1 *tat* splice-junction probe was confirmed by hybridization with a splice-junction control (SJ-C) probe. The SJ-C probe failed to detect any signals under the same experimental conditions (Fig. 6, c, f, and i), indicating that the separate but reversed exon components were unable to hybridize to any HIV-1 *tat* nucleotide sequence. In conjunction with the experiments presented below, these results suggest that splicing of HIV-1

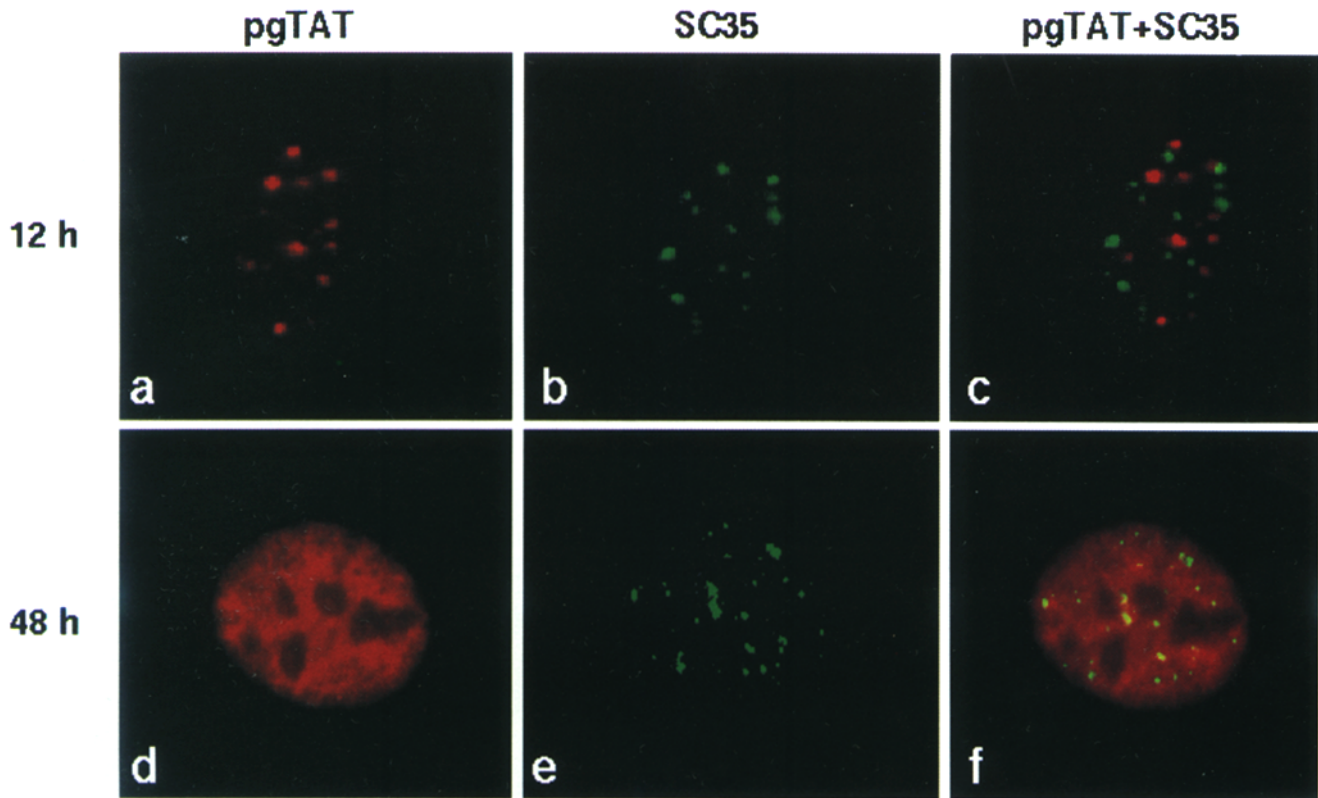


Figure 5. Distribution of HIV-1 *tat* RNAs relative to intranuclear speckles. The distribution of nascent and stable populations of HIV-1 *tat* RNA relative to the intranuclear speckles was examined by in situ hybridization with a *tat* intron-specific probe (*a* and *d*) and indirect immunofluorescence with an anti-SC35 antibody (*b* and *e*). Superimposition of the red and green signals appears as yellow (*c* and *f*).

tat RNA predominantly takes place posttranscriptionally, consistent with the presence of inefficient splicing sites of HIV-1 transcripts (Feinberg et al., 1986; Malim et al., 1988; Malim and Cullen, 1993). Furthermore, the failure to visualize a disperse nuclear distribution of spliced HIV-1 RNA suggests that, after splicing, the HIV RNA undergoes rapid export from the nucleus.

Evidence for Cotranscriptional Splicing of Efficiently Spliced Pre-mRNAs

To compare HIV-1 pre-mRNA processing to that of an efficiently spliced cellular pre-mRNA, we analyzed the human β -globin gene. HeLa cells were transfected with a human β -globin expression plasmid (pH β), and the distribution of unspliced and spliced β -globin RNAs was detected simultaneously by double-label fluorescence hybridization using globin-specific IN and SJ probes (Fig. 7). Unspliced human β -globin RNA was distributed predominantly as punctate nuclear dots at both early and late times after transfection. Consistent with our previous results with adenovirus and the cellular actin genes (Zhang et al., 1994), spliced globin RNAs were visualized in the nucleus as a number of punctate nuclear dots that colocalized with unspliced RNA at all times analyzed. In addition, spliced β -globin RNA was observed as a disperse distribution in the cytoplasm. A β -globin SJ-C probe failed to detect signals under the same experimental conditions (data not shown). These results suggest that, in contrast to the splic-

ing pattern of HIV-1 *tat* RNAs (Fig. 6), splicing of the human β -globin pre-mRNA occurred at the sites of transcription. Taken together, these results indicate that the efficiently spliced β -globin pre-mRNA undergoes cotranscriptional splicing, whereas the inefficiently spliced HIV pre-mRNA does not.

Discussion

The relationship between the expression of a gene and its intranuclear localization is an unresolved and much debated issue. HIV-1 Rev is one of the few regulatory proteins known to affect intracellular mRNA distribution, and therefore, expression of HIV genes provides an excellent model system to address this topic. Two key questions regarding Rev action are: (*a*) what is the mechanism that retains HIV pre-mRNAs in the nucleus in the absence of Rev? and (*b*) how does Rev promote the redistribution of these viral RNAs? The data presented in this study are relevant to both these questions.

Nuclear Compartmentalization and Rev Function

Previous studies have invoked a role for the nucleolus or intranuclear speckles in Rev function (Malim et al., 1989b; Nosaka et al., 1993; Kallard et al., 1994). These suggestions were based upon the apparent colocalization of Rev with these intranuclear structures. However, these earlier studies failed to localize the HIV-1 RNAs upon which Rev

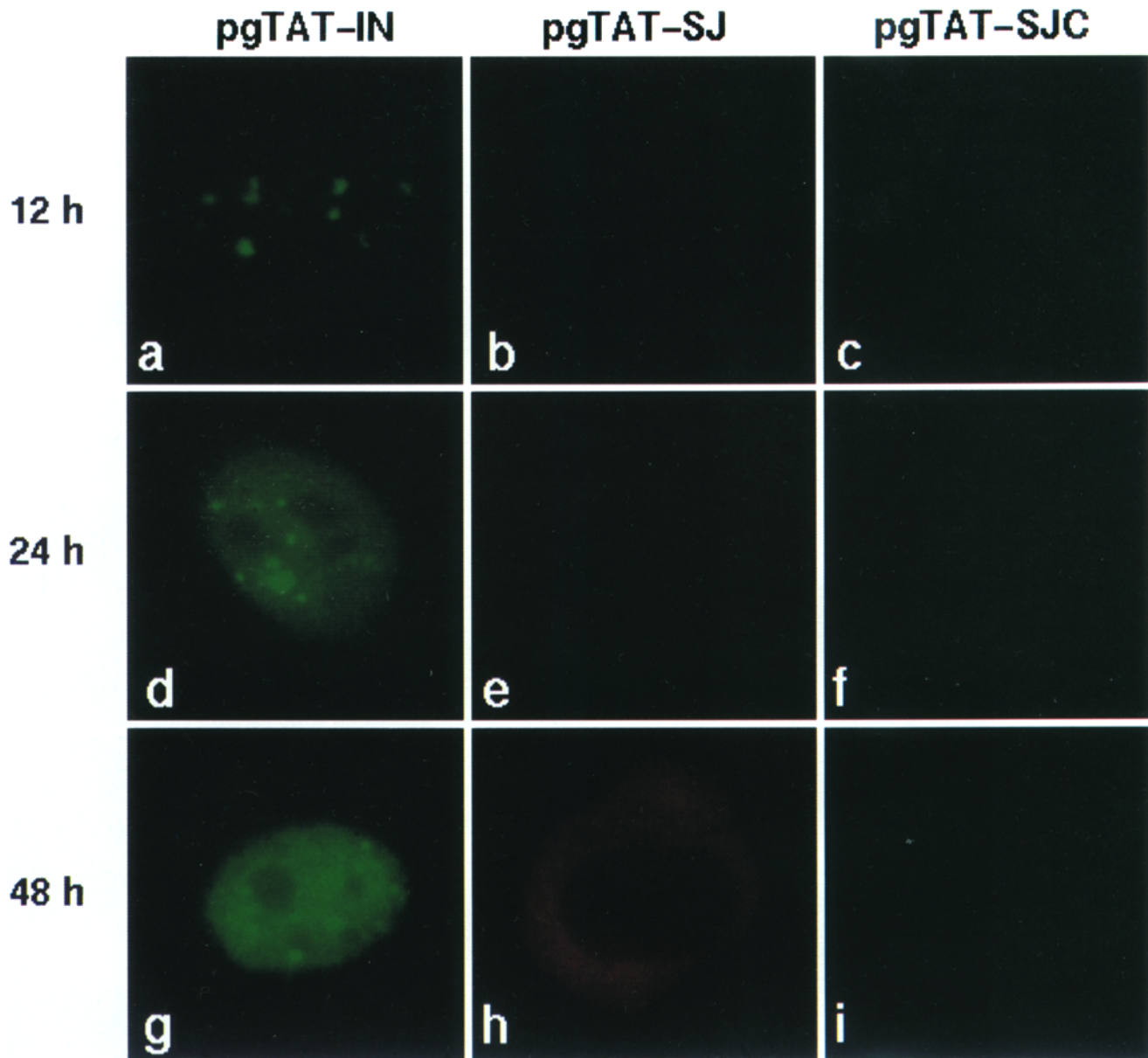


Figure 6. Spatial relationship of unspliced and spliced HIV-1 *tat* RNAs. The distribution of unspliced and spliced HIV-1 *tat* RNA was detected by in situ hybridizations with a *tat* intron (*IN*; *a*, *d*, and *g*), splice-junction-specific (*SJ*; *b*, *e*, and *h*), or a splice-junction control (*SJC*; *c*, *f*, and *i*) probe.

acts. Here, we have shown that the nuclear-localized, unspliced HIV-1 RNAs are randomly distributed throughout the nucleus. Thus, subnuclear structures such as speckles and the nucleolus are not involved in Rev-mediated nuclear RNA export. Our results are consistent with previous studies demonstrating that chimeric Rev derivatives were not localized to the nucleolus but retained Rev function (McDonald et al., 1992). We suggest that Rev's apparent nucleolar concentration is due to its ability to bind RNA nonspecifically at high concentrations and the high density of RNA in the nucleolus. The apparently random distribution of Rev-responsive HIV RNAs suggests that the components necessary for viral transcription, RNA biogenesis, and nuclear RNA export are available throughout the nucleoplasm. This conclusion is consistent with

several previous observations. First, immunolocalization experiments have shown that transcription factors (Spector, 1993), some pre-mRNA splicing factors such as U2AF (Zamore and Green, 1991) and U1 snRNA (Carmo-Fonseca et al., 1991), and hRIP/Rab, the likely Rev cellular cofactor (Bogerd et al., 1995; Fritz et al., 1995), are randomly distributed. Furthermore, even pre-mRNA splicing factors that have been reported to have a speckled distribution in the nucleus are also present, albeit at lower amounts, outside of the speckled domains (Spector, 1993). Second, our results are in agreement with several previous immunolocalization studies demonstrating that cellular transcription occurs throughout the nucleus with no evidence for compartmentalization. For example, a disperse distribution of nascent transcripts was detected by indirect immunofluo-

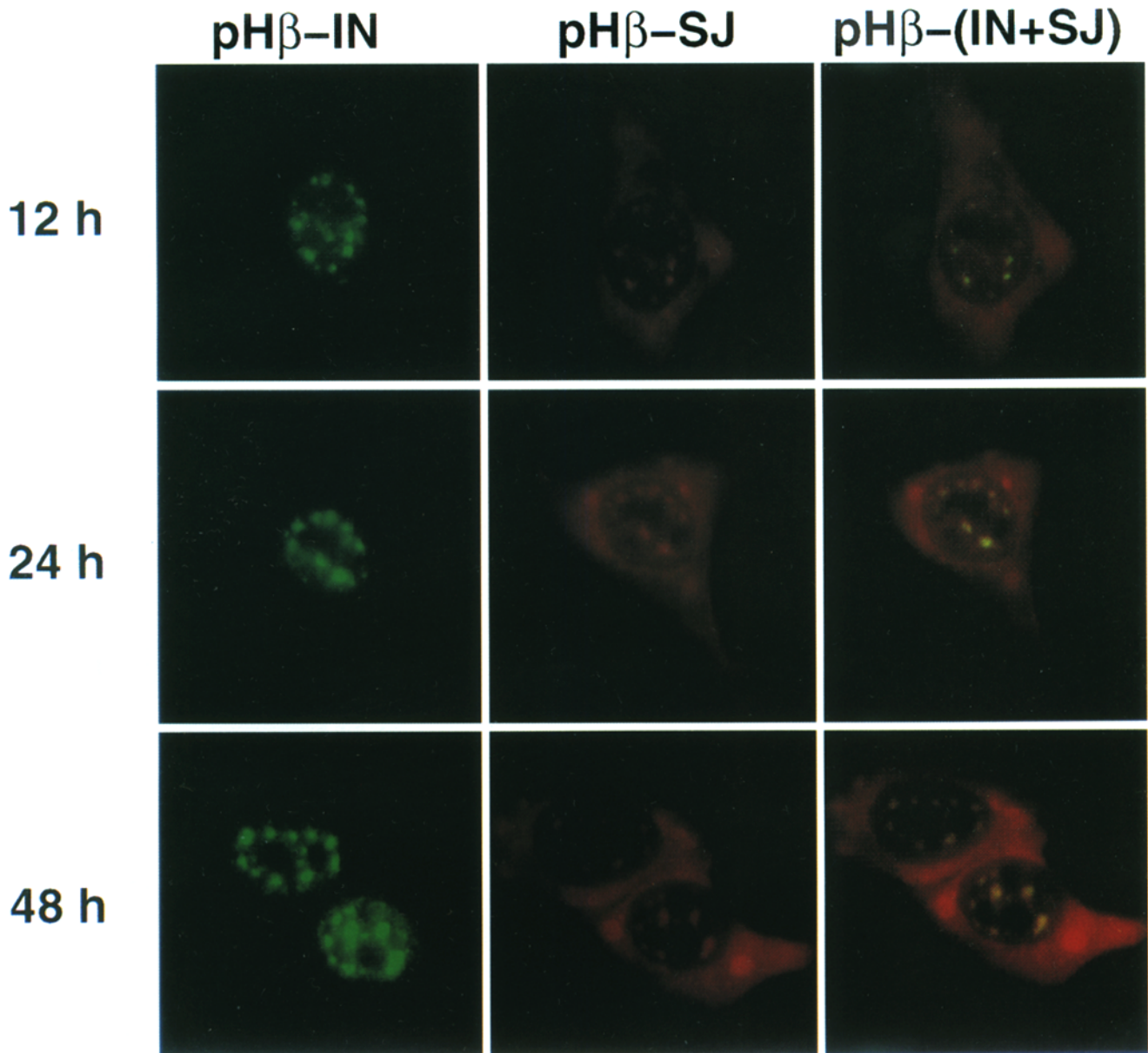


Figure 7. Intracellular distribution of unspliced and spliced human β -globin RNAs. In situ hybridization was performed using double-labeled human β -globin intron ($pH\beta$ -IN; *a*, *d*, and *g*) or splice-junction-specific ($pH\beta$ -SJ; *b*, *e*, and *h*) probes. The overlay of red and green signals appears as yellow.

rescence of HeLa cells pulse labeled with bromouridine triphosphate (Jackson et al., 1993; Wansink et al., 1993). These nascent transcription sites did not colocalize with speckles, yet, as mentioned above, pre-mRNA splicing can occur on nascent RNAs. Second, autoradiographic EM studies have shown that [3 H]uridine-labeled nascent transcripts are coincident with "perichromatin fibrils." In contrast, the speckles are congruent with "interchromatin granules," which are not major sites of RNA synthesis (Fakan et al., 1984). Finally, using an experimental approach similar to the one described here, we have shown previously that transcription and pre-mRNA splicing can occur throughout the nucleus with no evidence for compartmentalization (Zhang et al., 1994).

Cotranscriptional Splicing and Splice Site Strength

Previous work by several groups have demonstrated that splicing of eukaryotic pre-mRNAs can occur cotranscriptionally (Aebi and Weissman, 1987; Beyer and Osheim, 1989; LeMaire and Thummel, 1990; Baurén and Weislander, 1994; Zhang et al., 1994). Our studies have shown that whereas apparent cotranscriptional splicing can be detected for pre-mRNAs such as the adenovirus major late (Zhang et al., 1994) and human β -globin (this study), which have efficient splice sites, in a comparable experiment, cotranscriptional splicing of the inefficiently spliced HIV pre-mRNA was not detected. Together, these results suggest that transcription and pre-mRNA splicing are in-

dependent processes; efficient splice sites and long transcription units may favor cotranscriptional splicing, whereas inefficient splice sites and short transcription units would favor posttranscriptional splicing.

Basis for Nuclear Retention

The basis for the nuclear retention of Rev-responsive RNAs is still unclear. Our results clearly indicate that in the absence of Rev, compartmentalization in specific subnuclear regions is not the basis by which HIV-1 RNAs are retained in the nucleus. Spliceosome formation has been proposed to retain inefficiently spliced pre-mRNAs in the nucleus (Chang and Sharp, 1989; LeGrain and Rosbash, 1989). The suboptimal HIV-1 splice sites may allow partial spliceosome assembly, which would prevent nuclear RNA export. Consistent with this possibility, previous studies have shown that Rev regulation requires inefficient splice sites (Chang and Sharp, 1989; Lu et al., 1990; Kjems et al., 1991b; Malim and Cullen, 1993). Alternatively, negative *cis*-acting regulatory sequences within the HIV-1 pre-mRNAs have been suggested to play an active role in the nuclear retention of these RNAs (Dayton et al., 1989; Felber et al., 1990; Hammarskjold et al., 1991; Schwartz et al., 1991).

hRIP Distribution and Rev Action

The human nucleoporin-like protein, hRIP/Rab, which interacts directly with Rev's effector domain, is uniformly distributed throughout the nucleus with nucleolar exclusion (Fritz et al., 1995). Thus, the nuclear distribution of hRIP/Rab coincides with that of Rev and, as shown here, with the intranuclear distribution of HIV-1 RNAs upon which Rev acts.

Based on the results presented here and on previous studies, we propose the following as a working model for HIV RNA biogenesis. Due to inefficient splice sites, the HIV pre-mRNAs are released from the transcription template in an unspliced form. In the absence of Rev, these RNAs remain in the nucleoplasm either by an active retention mechanism or because they fail to engage the appropriate nuclear RNA export factors. The HIV-1 RRE-containing RNAs are bound by Rev, providing a recognition surface for hRIP/Rab. hRIP/Rab then binds to the Rev-RNA complex and is believed to promote subsequent protein-protein interactions required for recognition and translocation through the nuclear pore complex (Bogerd et al., 1995; Fritz et al., 1995; Stutz et al., 1995). We suggest that the similar intranuclear localizations of HIV RNA, Rev, and hRIP help to ensure that these interactions occur in an efficient and orderly fashion.

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