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cis-Acting Elements in Human Immunodeficiency Virus Type 1 RNAs Direct Viral Transcripts to Distinct Intranuclear Locations

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Two distinct intranuclear locations were identified for alternatively spliced RNA transcripts expressed from the pNL4-3 infectious molecular clone of human immunodeficiency virus (HIV) type 1. Multiply spliced HIV RNA encoding *tat* was detected within the nucleus in large clusters; immunostaining and colocalization studies using laser-scanning confocal microscopy revealed that these structures contained the non-small nuclear ribonucleoprotein RNA processing factor, SC35. In contrast, unspliced *gag* RNA was detected in much smaller granules distributed throughout the nucleus, with little or no association with SC35-containing granules. Analyses of nuclear RNA expressed from recombinant plasmids encoding *gag* (pCMVgag-2) alone or *tat* (pCMVtat-2) alone revealed distributions corresponding to those obtained with pNL4-3, indicating that expression within the context of the HIV provirus was not required for the distinct RNA locations detected for these transcripts. The presence of unspliced *gag* RNA in small granules was confirmed in infections of H9 T-lymphocytic cells, indicating that *gag* localization was not restricted to transient expression systems. The intranuclear distribution of *gag* RNA was dependent on specific RNA sequences. Deletion of a portion of the *gag* gene of pCMVgag-2, containing a *cis*-repressing inhibitory region, resulted in redirection of unspliced *gag* RNA from small granules into large SC35-containing clusters. The addition of the Rev-responsive element, RRE, to the deleted pCMVgag-2 construct resulted in RNA transcripts which were no longer associated with SC35. We also identified a cellular intron, rabbit β-globin-intervening sequence 2 (IVS-2) which, when introduced into pCMVgag-2, redirected unspliced *gag* RNA into SC35-containing granules and permitted *rev*-independent Gag expression. These findings suggest that redirecting intranuclear RNA localization may influence gene expression. Color micrographs from this article are available for view at http//128.231.216.2/lmmhome.htm.

Replication of human immunodeficiency virus type 1 (HIV-1) is regulated by transcriptional and posttranscriptional events, which require interactions between viral and host-encoded factors (48). The entire HIV-1 genome is expressed from a unique promoter located in the 5′ long terminal repeat (LTR) portion of the integrated provirus, and a 9.2-kb genome-length transcript is alternatively spliced, yielding numerous transcripts encoding HIV-1 gene products (4, 24, 53, 58, 63, 66). Regulation of differential HIV RNA processing is complex; expression of unspliced and singly spliced RNA species requires binding of the HIV-1 regulatory gene product, Rev, to a highly structured RNA target, the Rev-responsive element (RRE), while expression of multiply spliced 1.8-kb RNA occurs in the presence or absence of *rev* (5, 11, 16, 17, 25, 26, 28, 29, 44–47). Additional *cis*-acting sequences within the HIV-1 *gag/pol* and *env* genes which inhibit their own expression are reported to participate in regulation of RNA expression as well. These sequences, denoted instability sequences (INS [52, 64, 65]), *cis*-repressor sequences (CRS [12, 15, 61]), or inhibitory regions (IR [42]), all share the requirement for the RRE sequence in *cis* and *rev* in *trans* for full expression in the cytoplasm. The nature of the inhibitory block conferred by repressor sequences and the mechanisms by which *rev* relieves *cis* inhibition remain unknown. Recently, *rev*-independent *gag* expression has been achieved by a series of conservative base substitutions which interrupt AT-rich regions (64), and *rev*-independent *env* expression was observed when the *env* gene was positioned downstream of an efficiently spliced β-globin intron (IVS-2) and when cryptic splice sites within *env* were removed, redefining the *env* gene as an exon (27).

In previous RNA fractionation experiments, we reported that HIV inhibitory sequences were localized predominantly to the nucleus (42). In the present communication, we investigated the intranuclear location of HIV RNA sequences by using in situ hybridization and confocal microscopic techniques. HIV RNA species were detected in two distinct intranuclear locations; a multiply spliced RNA species encoding the *tat* gene was identified in nuclear speckles and colocalized with the RNA processing factor SC35. In contrast, unspliced RNA was identified in small granules which did not colocalize with SC35. Further analysis revealed that the presence of the RRE or inhibitory *gag* sequences in *cis* was capable of directing RNA localization within the nucleus. In addition, we identified that the β-globin (IVS-2), which has been shown to enable *rev*-independent *env* expression (27), redirected HIV *gag* RNA into SC35-containing granules and allowed *rev*-independent *gag* expression. These findings indicate that *cis*-acting sequences can direct the intranuclear location of RNA transcripts and suggest that physical compartmentalization within the nucleus may influence HIV RNA processing and gene expression.

MATERIALS AND METHODS

Cells and transfection. HeLa cells were maintained in DMEM supplemented with 10% fetal bovine serum, glutamine, and antibiotics (maintenance medium) (70). Uninfected H9 cells and H9 cells chronically infected with HIV IIIB (H9IIIB) or with HIV pNL4-3 (H9NL43) were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, glutamine, and antibiotics (43). HIV

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infections were initiated by cell-cell transmission as previously described (43, 62) and examined 21 h after cocultivation. HeLa cells were transfected with 20 μg of plasmid DNA, as previously described (70), except that the cells did not undergo glycerol shock. For in situ hybridization and/or immunofluorescence studies, transfected cells were washed, scraped, and reseeded into 12-well plates 4 to 5 h after DNA addition. T-lymphocytic cells were attached to glass slides by cytocentrifugation or by coating slides with poly-lysine. For RNA analyses, cells were washed and refed with maintenance medium 4 to 5 h after DNA addition and harvested 24 to 48 h later. In experiments comparing Gag protein expression, plasmids were cotransfected with pCMV-CAT as an internal control; lysates prepared from these transfections were normalized for transfection efficiency by measuring chloramphenicol acetyltransferase enzyme activity (42), and then p24 determinations were performed.

Plasmids. The pNL4-3 infectious molecular clone of HIV-1 (1), *tat*-expressing pCMV-tat-2 (22), *rev*-expressing pCMV-rev (2), pCMV-CAT (2), and rabbit β-globin-expressing pAL4-SV (9) have been previously described. A *rev*-defective derivative of pNL4-3, pNL4-3*rev*$^-$, was constructed by restricting it with *Bam*HI, extending the recessed strand with the Klenow fragment of Pol I, and religating the blunt ends with DNA ligase. Recombinant plasmids expressing portions of the HIV-1 *gag*/*pol* gene from the cytomegalovirus immediate-early promoter were constructed by a multistep strategy. The *Bgl*II restriction fragment (nucleotide [nt] 679 to 2096, numbered according to the pNL4-3 entry in reference 51) from pNL4-3 containing the HIV-1 *gag* p17, p24, and a portion of p9 was ligated to *Bam*HI-restricted, calf intestinal phosphatase-treated pCMV-CAT vector, yielding pCMVgag-0. To provide the HIV LTR polyadenylation signal, the *Eco*RV-*Eco*RI fragment containing the HIV LTR from the pNLA-1 plasmid (69) was ligated to pCMVgag-0 restricted with *Sma*I-*Eco*RI, generating pCMVgag-3. Sequences 3′ to the LTR were removed by restricting pCMVgag-3 with *Eco*RI and *Nar*I, extending the recessed strand with Klenow Pol I, and religating the blunt ends, yielding pCMVgag-2 (see Fig. 6). An RRE-containing derivative of pCMVgag-2 was constructed by inserting the RRE into a unique *Bgl*II site of pCMVgag-2. A DNA fragment (nt 7721 to 7993) including the RRE was obtained by PCR, amplifying pNL4-3 with oligonucleotides containing the *Bgl*II recognition sequence at the 5′ end: 5′ GAAGATCTTCGAAGAGTGGT GCAGA 3′ and 5′ CCTAGTTGTCGAGGAGGTCTAGAAG 3′; the amplified DNA fragment was restricted with *Bgl*II and ligated to the *Bgl*II-restricted, calf intestinal phosphatase-treated pCMVgag-2 (see Fig. 6). Deletion mutants of pCMVgag-2 and pCMVgag-2RRE were obtained by restricting each plasmid with *Acc*I and religating, generating pCMVgag-2ACC and pCMVgag-2ACCRRE, respectively (see Fig. 6). A derivative of pCMVgag-2 containing the second intervening sequence of rabbit β-globin upstream of the major splice donor was constructed by obtaining the *Bam*HI-*Eco*RI fragment from rabbit β-globin plasmid pAL4-SV, extending the recessed strands with Klenow, and blunt-end ligating to *Bss*HII-restricted, blunt-ended, phosphatase-treated pCMVgag-2. The structures of the recombinant clones were analyzed by restriction digests, and the sequences of the relevant portions of the constructs were confirmed by dideoxy sequencing.

In situ hybridization. DNA restriction fragments, whole plasmids, or PCR-amplified DNAs were used to specifically detect RNA by hybridization as described by Johnson et al. (32). HeLa cells on glass coverslips were harvested 24 to 48 h after transfection; coverslips were washed two to three times with Dulbecco's phosphate-buffered saline (PBS) and fixed at room temperature (RT) for 10 min in 4% paraformaldehyde–PBS buffered to pH 7.4. Coverslips were washed two times with PBS and stored in 70% ethanol at 4°C. For hybridization, the probe was redissolved in 120 μl of 83% formamide containing 0.83 mg of tRNA per ml and 0.83 μg of sheared salmon sperm DNA per ml, heated to 70°C for 10 min, and then immediately chilled. The probe was mixed 1:1 with hybridization buffer (4× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.4% bovine serum albumin [BSA], 0.4 mM vanadyl riboside complexes, 20% dextran sulfate), and 20 μl of this mixture was applied to each coverslip. The coverslips were covered with parafilm and incubated at 37°C for 3 h overnight in a humidified chamber. After hybridization, cells were washed sequentially in 50% formamide in 2× SSC at 37°C for 20 min, 2× SSC at 37°C for 20 min, 1× SSC at RT for 20 min, and 4× SSC at RT for 10 min. The hybridized probes were detected by incubation with Texas red-conjugated streptavidin (Molecular Probes, Eugene Ore.; 2 μg/ml in 4× SSC–1% BSA) for 30 min to 1 h at 37°C, washed three times with 4× SSC for 10 min at RT, and mounted onto glass slides in Fluormount (Virotech International, Rockville, Md.). For colocalization experiments, coverslips were incubated in PBS–1% BSA at 37°C for 30 min with specific mouse monoclonal antiserum SC35 (gift of T. Maniatis [21]), human serum from an HIV-seropositive individual, mouse monoclonal anticoilin (gift of E. Chan), rabbit anti-Rev (gift of G. Pavlakis), mouse monoclonal anti-p24 (New England Nuclear), or fluorescein isothiocyanate-conjugated mouse monoclonal anti-p24 (Seramun). The sera were visualized by incubating with the corresponding fluorescein-conjugated goat anti-mouse, goat anti-human, or goat anti-rabbit serum.

The cells were examined by laser-scanning confocal microscopy with a Zeiss LSM 4 confocal microscope system (Carl Zeiss, Thornwood, N.Y.). A 488-nm incident beam from an Ar-Kr laser and a 515-565 band-pass filter were used to detect fluorescein, and a 568-nm incident beam and 590-nm long-pass filter were used to identify the Texas red signal; the 568-nm incident beam and Nomarski optics were employed to obtain a light microscopic image. The fluorescein, Texas red, and Nomarski images were stored in separate channels as black and white images; to determine colocalization, the RNA image (pseudocolored red) and the SC35 immunofluorescence image (pseudocolored green) were electronically recombined; the colocalized image is pseudocolored yellow-orange; colocalization was confirmed in all cases by using the colocalization function of the LSM-4 software package. Where no colocalization was detected, the entire cell was optically sectioned, either manually or automatically, to ensure that no significant areas of colocalization were present. At least four independent transfections were performed to compare the constructs, and several hundred total cells were analyzed for each transfected plasmid. No colocalization studies were possible using in situ hybridization and anti-Rev sera, because Rev reactivity was lost after the in situ hybridization procedure. Color micrographs from this article are available for view at http//128.231.216.2/lmmhome.htm.

RNA analyses. Whole-cell RNA preparations were obtained by guanidinium extractions as described previously (42), or polyadenylated RNA was isolated from transfected HeLa cells using an Invitrogen fast track mRNA purification kit (Invitrogen, San Diego, Calif.). RNA was electrophoresed in 1% agarose-formaldehyde gels, transferred to nitrocellulose, and hybridized to a ^{32}P-end-labeled oligonucleotide complementary to the major splice donor region of pNL4-3 (5′ CCCATCTCTCTCCTTCTAGCCTCCGCTAGTCAAAATTTTTGGCGTACT CACCAGTCGCCGCCCCTCGCCTCTTGCCGTGCGC 3′), as previously described (42).

Probe preparation. DNA probes were prepared from restriction fragments (probe ACC, *Acc*I position 959-*Acc*I position 1678) from PCR fragments (probe D, position 736 to 959 using oligonucleotide primers 5′ GGCGACTGGTGAG TACGCCAAAAATT 3′ and 5′ CAGCCTTCTGATGTCTCTAAAAGGACC 3′; probe E, position 769 to 2011 using oligonucleotide primers 5′ GCGGAG GCTAGAAGGAGAG 3′ and 5′ GGGCCCTGCAATTTTTGGC 3′; probe RRE, position 7721 to 7993 using oligonucleotide primers used above to amplify the RRE for cloning), or from entire plasmids (pCMVTAT-2 and pNL4-3) as noted by Johnson et al. (32). Probes were labeled by incorporation of biotin 14-dATP in nick translation reactions using the BIONICK labeling system (GIBCO Life Sciences, Gaithersburg, Md.) under conditions stipulated by the supplier. Each probe preparation was mixed with 10 μg of sheared salmon sperm DNA–1/10 volume 3M sodium acetate and then precipitated with 3 volumes of 95% ethanol and centrifuged, and the pellet was washed in 70% ethanol. To detect polyadenylated RNA, a poly(T) (64-mer) oligonucleotide was synthesized containing a biotinylated dT at every fourth residue.

p24 determination. Quantitative p24 antigen levels in extracts of transfected cells were measured with the Coulter HIV-1 p24 antigen capture assay kit (Coulter Corp., Hialeah, Fla.).

RESULTS

Two distinct patterns of HIV-1 RNA intranuclear distribution. HIV RNA processing results in three distinct size classes of viral RNA, 9.2, 4.5, and 1.8 kb in size. We investigated the intracellular distribution of the different HIV RNA species by fluorescent in situ hybridization (FISH) and confocal microscopy. HIV RNA transiently expressed in HeLa cells transfected with the infectious molecular clone pNL4-3 was hybridized in situ to complementary biotinylated DNA probes (Fig. 1). Hybridized probes were detected by staining with Texas red streptavidin, and the cells were examined by laser-scanning confocal microscopy. As shown in Fig. 2, hybridization to *gag* probe ACC (see Fig. 1 for position) revealed that unspliced HIV RNA was present in the cytoplasm and was distributed within the nucleus in small granules finely stippling the nucleoplasm but was excluded from the nucleolus (Fig. 2c). RNA-expressing cells were actively producing HIV protein in the cytoplasm; immunostaining with anti-p24 antisera (Fig. 2a) revealed that all cells expressing HIV RNA were producing p24^{Gag} protein. Hybridization with biotinylated probes was specific for HIV RNA; after RNase digestion, p24-expressing cells (Fig. 2b) no longer contained any hybridization signal (Fig. 2d). The distribution of HIV unspliced RNA in small granules within the nucleus was also demonstrated with a second probe, D (Fig. 1) (data not shown). FISH using a probe, RRE (Fig. 1), complementary to the Rev-responsive element contained within the *env* gene, also revealed a finely stippled pattern of RNA in the nucleus (Fig. 3b), similar to that detected with a probe ACC complementary to unspliced HIV RNA (Fig. 3a). Since both unspliced *gag* and singly spliced *vif* and *vpu*/*env* RNA transcripts contain the RRE sequence, it is

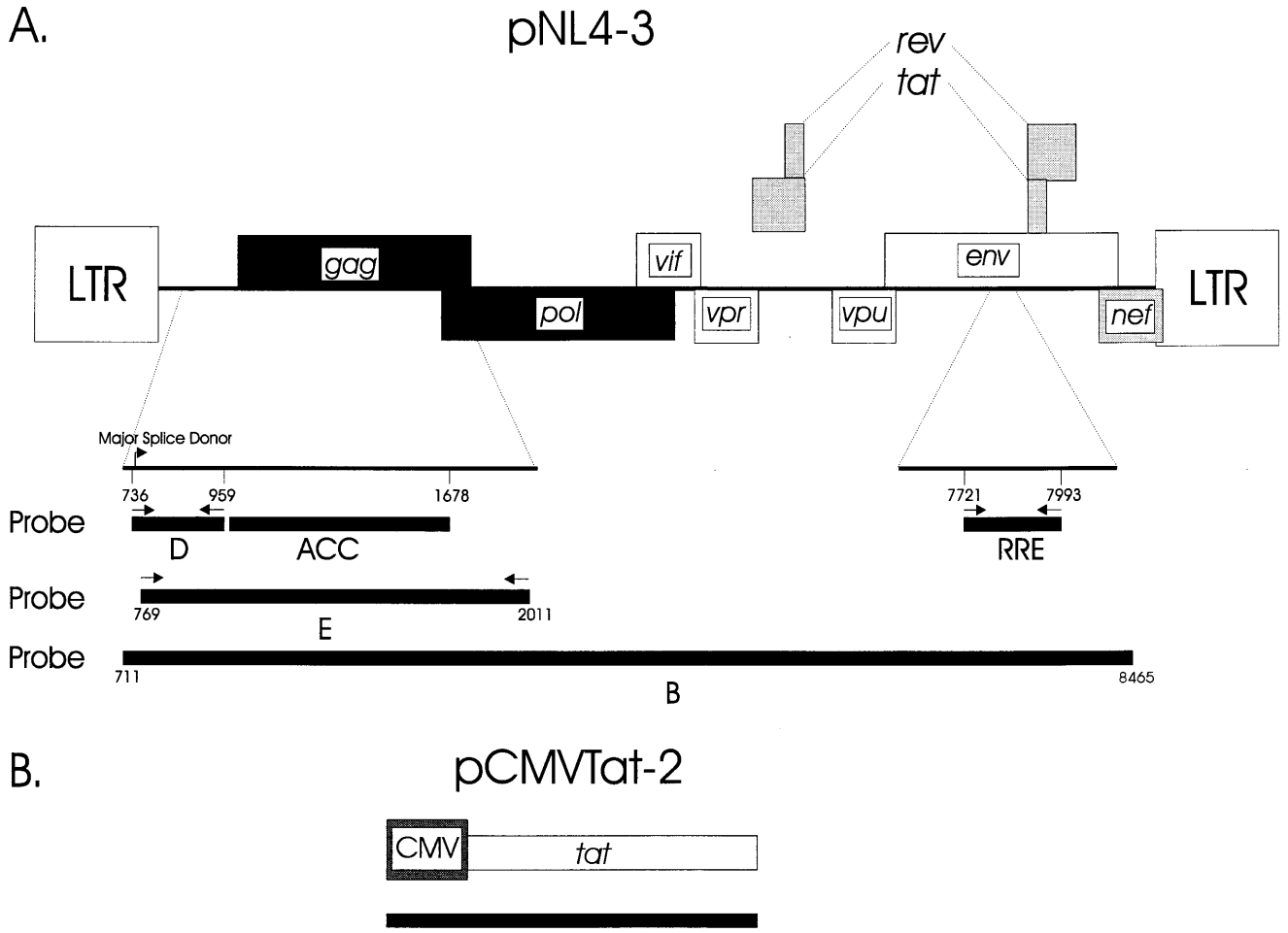

FIG. 1. Schematic of probes used for HIV in situ hybridization. (A) The pNL4-3 infectious molecular clone of HIV is shown, coded to illustrate genes expressed from unspliced (black), singly spliced (white), and multiply spliced (gray) mRNA transcripts. Biotinylated probes were prepared from an *Acc*I restriction fragment (ACC), *Bss*HII-*Eco*RI + *Eco*RI-*Bam*HI restriction fragments (B), or DNA fragments of the *gag* (D and E) and *env* (RRE) gene amplified by PCR, as described in Materials and Methods. (B) pCMVtat-2 is a cDNA clone of HIV-1 *tat* under the transcriptional control of the cytomegalovirus immediate-early promoter (22). A biotinylated probe was prepared from the plasmid as described by Johnson and coworkers (32).

not possible to determine whether the RRE hybridization signal corresponds to unspliced or singly spliced transcripts. However, because the hybridization signal obtained with the RRE probe was present in the nucleus as a uniformly fine stippling, we infer that both unspliced and singly spliced HIV RNA transcripts were present in small granules.

In contrast to the distribution of unspliced and singly spliced transcripts, the majority of transcripts encoding the multiply spliced *tat* gene accumulated in fewer, larger clusters of approximately 2 μm in size distributed in the nucleus exclusive of the nucleolus (Fig. 3c). A small amount of the RNA hybridizing to the *tat* probe was present in small granules as well (Fig. 3c); it is likely that some of this stippled signal includes unspliced, full-length RNA identified by the *tat* probe. These findings suggest that the sites in the nucleus where HIV unspliced and multiply spliced RNAs accumulate are distinct.

To characterize the intranuclear locations of HIV RNA species, we immunostained cells after hybridization with antisera elicited against the non-small nuclear ribonucleoprotein splicing factor, SC35, which is organized into distinct units ("speckles") (67) within the nucleoplasm (Fig. 3d, e, and f). RNA hybridization and the SC35 immunostaining images of a field of cells were obtained by laser-scanning confocal microscopy and were recombined to determine whether HIV RNA and SC35 colocalized in the same nuclear compartment. Combining the in situ hybridization signal (Fig. 3a) with the corresponding immunostaining image (Fig. 3d) revealed that little colocalization of HIV unspliced *gag* RNA and SC35 was present in HeLa cells transfected with pNL4-3, indicating that the vast majority of steady-state *gag* RNA did not associate with SC35-containing clusters (Fig. 3g; RNA signal is pseudocolored red, SC35 signal is pseudocolored green, and colocalizing signal is yellow). Optical sectioning through entire transfected cells confirmed the presence of *gag* RNA exclusively in small granules; the small amount of *gag* signal which did colocalize with SC35 (Fig. 3g, yellow) did not conform to the contour of SC35-containing speckles but appeared to be a completely random association. Similarly, combining the hybridization signal obtained with probe RRE (Fig. 3b) with the SC35 immunostaining image (Fig. 3e) revealed little specific association of HIV RNA and SC35-containing speckles (Fig. 3h; RNA is pseudocolored red, SC35 is pseudocolored green, and colocal-

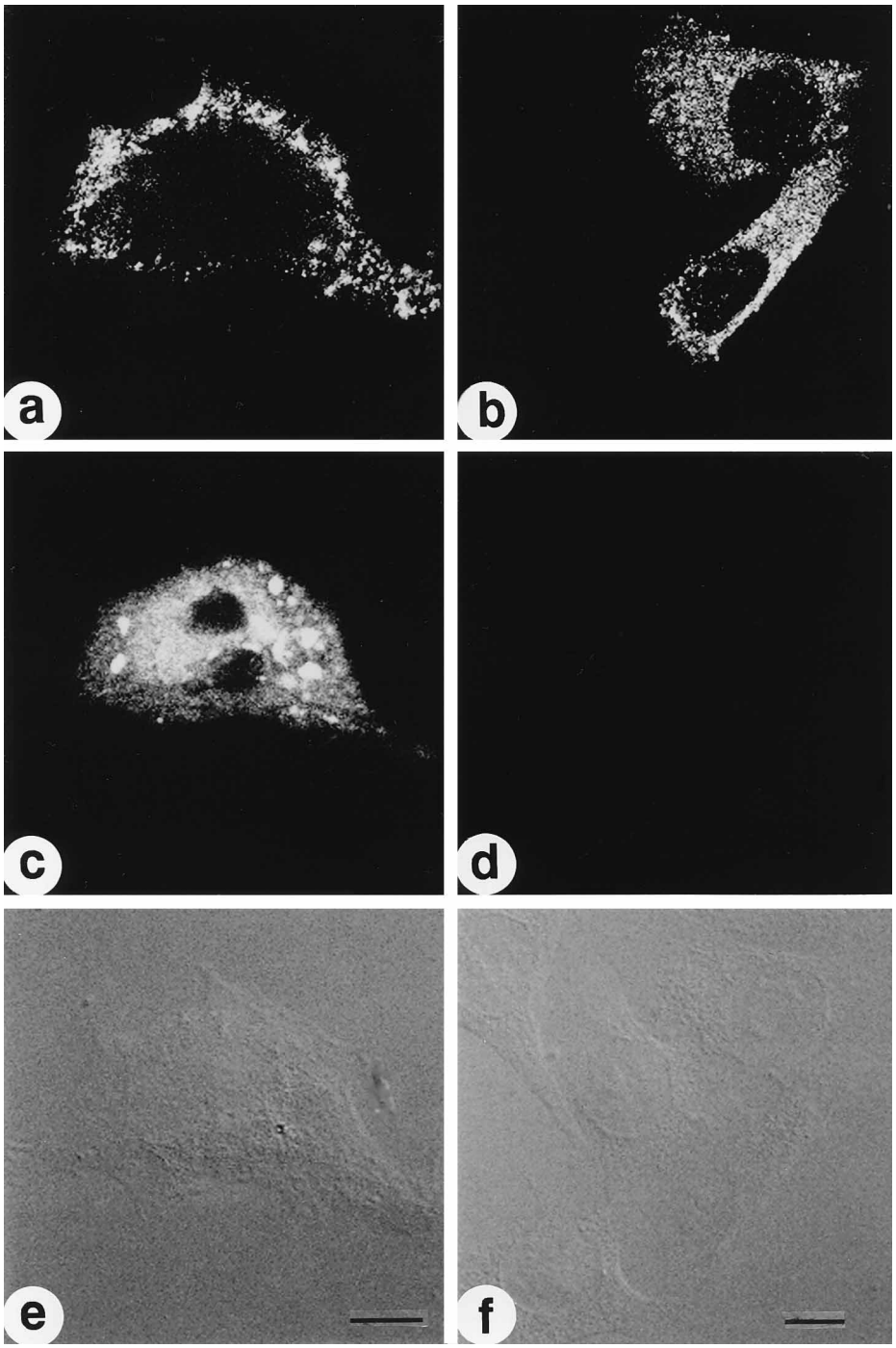

FIG. 2. In situ detection of HIV unspliced HIV mRNA and $p24^{Gag}$ protein. HeLa cells transfected with the infectious molecular clone pNL4-3 were fixed, treated in the absence (a, c, and e) or presence (b, d, and f) of RNase as described in Materials and Methods, hybridized to biotinylated DNA probe ACC (see Fig. 1 for position), and immunostained with antibody to p24. The hybridization (c and d), immunofluorescence (a and b), and Nomarski (e and f) signals of a field were imaged separately by laser-scanning confocal microscopy and individually stored as black and white images as described in Materials and Methods. In cells prepared in the absence of RNase treatment, a representative example demonstrates that p24 expression (a) and *gag* RNA expression (b) occur in the same cell (Nomarski, c). RNA was present in the nucleus and cytoplasm but was excluded from the nucleolus. After RNase digestion, p24-expressing cells (b) no longer contained *gag* RNA (d); the Nomarski image identifies several nontransfected cells with no detectable p24 immunostaining. Bar = 10 μm.

ization is yellow). Again, optical sectioning revealed no specific correlation between the RNA and the protein signal. These data suggest that neither unspliced nor singly spliced HIV RNAs accumulated in SC35-containing nuclear speckles.

In contrast to the findings with unspliced or singly spliced HIV RNA, combining the hybridization signal obtained with the *tat* probe (Fig. 3c) with the corresponding SC35 immunostaining image (Fig. 3f) revealed a high degree of colocalization (Fig. 3i; RNA is pseudocolored red, SC35 signal is pseudocolored green, and colocalizing signal is yellow). Although *tat* RNA was highly concentrated in some SC35-containing granules, other SC35-containing granules contained little *tat* RNA,

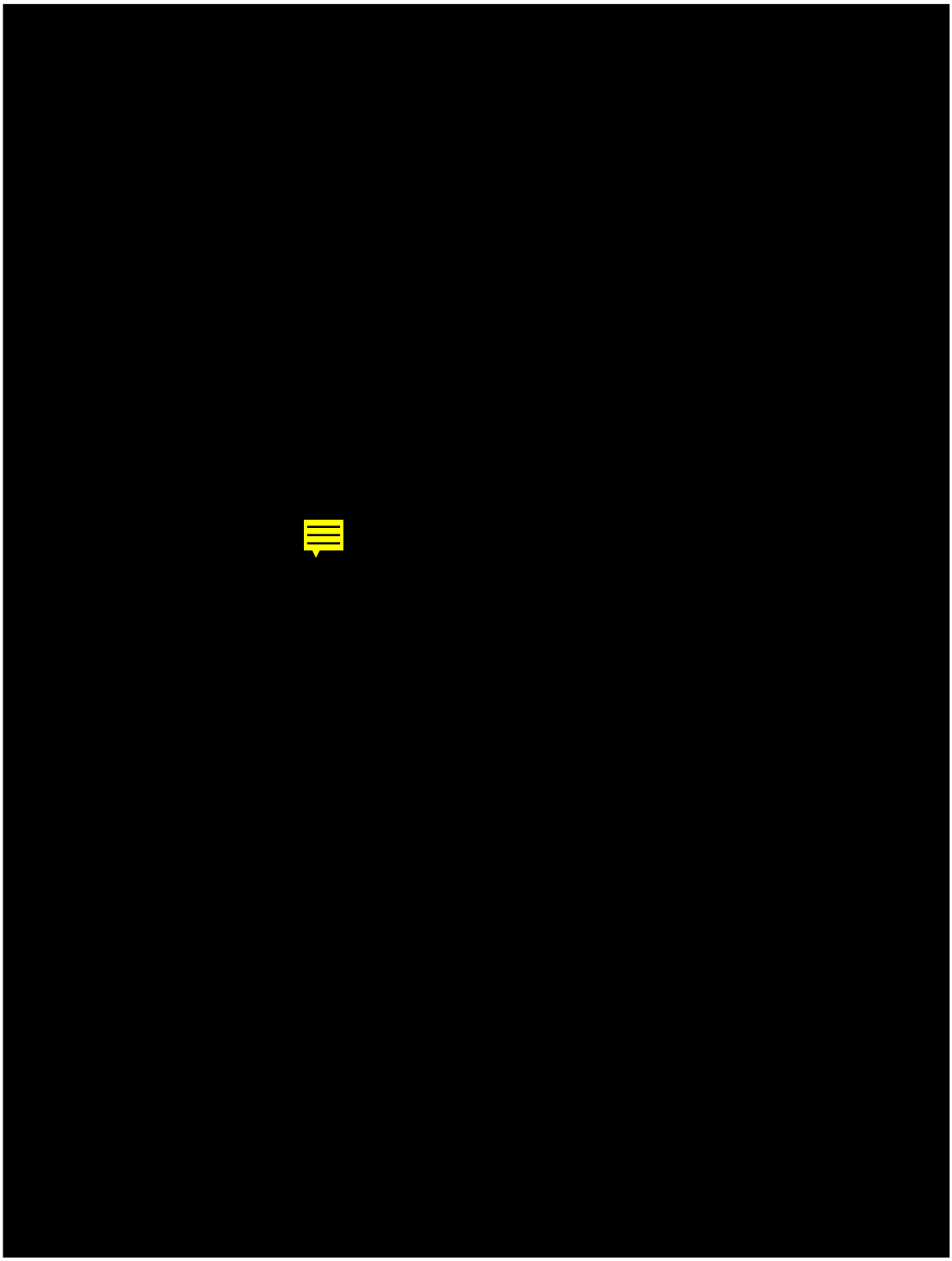

FIG. 3. Comparison of intranuclear distributions of unspliced and spliced HIV RNA. HeLa cells were transfected with pNL4-3, fixed, hybridized to biotinylated DNA probes complementary to unspliced (left column), singly spliced (middle column), and multiply spliced (right column) HIV RNA species, and immunostained with anti-SC35. Images of in situ hybridization (a to c), SC35 (d to f), and Nomarski imaging (j to l) were obtained. The in situ and immunostaining images were recombined (g to i) to determine colocalization; RNA is pseudocolored red, SC35 is pseudocolored green, and colocalization is yellow-orange. A representative cell showing hybridization with *gag* probe ACC (a) and SC35 staining (d) was recombined (g), revealing little colocalization between the unspliced *gag* RNA and SC35. Similarly, recombining the hybridization signal obtained using probe RRE complementary to *env* RRE sequence (b) with the corresponding SC35 immunostaining image (e) revealed little specific colocalization (h). In contrast, the distribution of multiply spliced RNA complementary to the *tat* probe (c) was similar to the distribution of SC35 immunostaining (f) and a high degree of colocalization (i, yellow-orange) was observed. Bar = 10 μm.

suggesting that these granules may not all be equivalent in accumulating *tat* RNA. Colocalization of multiply spliced HIV RNA species with SC35 in large clusters did not require expression within the context of the HIV provirus; colocalization of *tat* RNA and SC35-containing speckles was also observed in HeLa cells transiently expressing *tat* RNA from the cDNA expression plasmid pCMVtat-2 or from subviral expression constructs under the direction of the HIV promoter and was detected using probes made from HIV- or CMV-*tat* expression constructs (data not shown). Complete or partial SC35-RNA association has been described for the majority of RNA signals or tracks and for specific endogenous RNAs as well (reviewed in reference 67). Consistent with such observations, we detected colocalization of SC35 with the majority, but not all, of the cellular polyadenylated RNA signal obtained using an oligo(dT) probe (data not shown).

These studies suggest that, in steady-state conditions, alternatively spliced products of a single 9.2-kb HIV RNA transcript accumulate in distinct nuclear locations. Multiply spliced RNAs associate with large SC35-containing clusters, typical of endogenous RNA, whereas unspliced and singly spliced HIV RNA transcripts rarely colocalize with SC35-containing nuclear speckles. The presence of unspliced RNA clearly distinct from SC35 suggests a less common RNA distribution, similar to that described for a minority of polyadenylated cellular RNA species (8). We confirmed the uncommon RNA nuclear localization for HIV unspliced RNA in T-lymphocytic cells infected with HIV. FISH was used to investigate the distribution of unspliced HIV RNA in cultures of H9 cells chronically infected with HIV IIIB (H9IIIB) or HIV pNL4-3 (H9NL43). FISH of H9IIIB revealed unspliced HIV RNA present in the nucleus in small granules (Fig. 4, H9IIIB), similar in size that detected for unspliced HIV RNA in transient expression experiments in HeLa cells (compare Fig. 4, H9IIIB, with Fig. 3a). In contrast, uninfected H9 cells contained only a background fluorescence signal (Fig. 4, H9). Since coculture of uninfected H9 cells with H9 cells chronically infected with HIV initiates rapid cell-cell fusion and a well-defined cycle of HIV transmission, replication, and apoptosis (43), we used the cocultivation technique to obtain large numbers of newly infected cells and syncytia at a time during replication (21 h of coculture) when virus RNA production is high and before apoptotic nuclear destruction is extensive. As shown in Fig. 4, FISH of unspliced HIV RNA in nuclei of syncytia was present in numerous small granules (Fig. 4a); costaining with SC35 (Fig. 4b) and overlay of the in situ signal (Fig. 4c, red) and immunofluorescent signal (Fig. 4c, green) revealed only occasional colocalization between HIV unspliced RNA and SC35. These findings reveal that unspliced HIV RNA expressed after infection was present in areas of the nucleoplasm independent of SC35-containing granules, in a distribution similar to that observed in HeLa cells transiently expressing HIV.

To determine whether the intranuclear distribution of HIV *gag* RNA was dependent on expression of the *rev* gene product, we compared HIV RNA expression in HeLa cells transfected with *rev*+ and *rev*-defective pNL4-3. As shown in Fig. 5, unspliced *gag* RNA expressed from *rev*− pNL4-3 was present in the nuclei but not in the cytoplasm of transfected cells (Fig. 5b), whereas unspliced *gag* RNA expressed from *rev*+ pNL4-3 was present in the nucleus and cytoplasm of transfected cells (Fig. 5a). Unspliced intranuclear *gag* RNA was present in small granules in both rev+ and rev− pNL4-3 transfected cells, although the number of granules containing HIV RNA was often greater in the absence of Rev (compare Fig. 5a and b). Little colocalization of *gag* RNA expressed from *rev*-defective pNL4-3 with SC35-containing speckles was detected in the composite (Fig. 5f) obtained by combining the in situ hybridization image (Fig. 5b) with the corresponding SC35 immunostaining image (Fig. 5d); as expected, a similar absence of colocalization was observed in the composite (Fig. 5e) obtained by combining the in situ hybridization image (Fig. 5a) with the SC35-immunostaining image (Fig. 5c) for the *rev*+ pNL4-3. These results suggest that *rev* expression does not appreciably alter the intranuclear appearance of unspliced HIV RNA and suggests that localization may be an intrinsic property of the RNA transcripts themselves.

In addition to a nuclear distribution, unspliced HIV RNA was also identified in the cytoplasm of many transfected cells (compare cytoplasmic RNA in Fig. 2c, 3a and b, and 5a), often in large collections of 3- to 5-μm size exhibiting intense fluorescence. Cytoplasmic staining was specific; RNA accumulations were abolished after RNase treatment (Fig. 2d) and were not present in cells transfected with the *rev*− pNL4-3 clone (Fig. 5b). Large cytoplasmic accumulations of RNA are not restricted to virus-encoded genes but may be characteristic of transient expression, since we also detected large cytoplasmic accumulations of globin RNA in cells tranfected with the β-globin-expressing pAL4-SV plasmid (data not shown).

Nuclear RNA localization in a *gag* reporter construct. To investigate potential *cis* determinants of the intranuclear localization of HIV *gag* RNA, we constructed a subgenomic *gag*-expressing plasmid, pCMVgag-2 (Fig. 6), which exclusively expresses the HIV-1 *gag* gene independently of the HIV LTR. Analysis of pCMVgag-2 RNA expression by Northern blotting revealed two distinct RNA species, a 1,900-nt species corresponding to full-length transcript and a more abundant shorter transcript of approximately 900 nt (data not shown). The shorter transcript is likely to be the product of splicing to cryptic splice acceptors within the 3′ portion of the *gag* gene (5a). In situ hybridization using probe ACC in HeLa cells transfected with pCMVgag-2 revealed the presence of unspliced *gag* RNA in small granules randomly distributed throughout the nucleoplasm exclusive of the nucleolar region (Fig. 7a). Using a probe generated from the entire pCMVgag-2 plasmid to detect spliced and unspliced RNA, we identified a strong hybridization signal in the cytoplasm of pCMVgag-2-transfected cells, corresponding to the exported spliced RNA (Fig. 7b). The fact that spliced RNA is present in the nucleus and cytoplasm while unspliced RNA is localized exclusively to the nucleus suggests that nuclear sequestration of unspliced *gag* RNA was a specific consequence of intragenic sequences.

The intranuclear location of unspliced *gag* RNA was further investigated by using FISH-SC35 immunostaining colocalization studies. As shown in Fig. 8, unspliced RNA expressed from pCMVgag-2 was present in small granules (Fig. 8a), a pattern which was distinct from the distribution of SC35-containing speckles (Fig. 8d); colocalization studies (Fig. 8g) revealed no colocalization of unspliced *gag* RNA expressed from pCMVgag-2 and nuclear speckles, similar to the distribution of unspliced *gag* RNA expressed from pNL4-3 (compare Fig. 8a with Fig. 2c, 3a, and 5a). In addition, no colocalization of unspliced HIV RNA from pCMVgag-2 was detected by using antisera to another nuclear protein, coilin, which is a constituent of the RNA-containing coiled bodies (data not shown). These data indicate that the intranuclear distribution of unspliced *gag* RNA appeared similar whether the RNA was expressed within the context of the HIV provirus or as a simple expression plasmid under transcriptional control of a heterologous promoter. Because the pCMVgag-2 plasmid contains inhibitory sequences but does not contain the RRE sequence in *cis* and is not coexpressed with Rev, no unspliced *gag* RNA was detected in the cytoplasm of transfected cells using a probe complementary to the unspliced *gag* RNA (Fig. 8a); similarly,

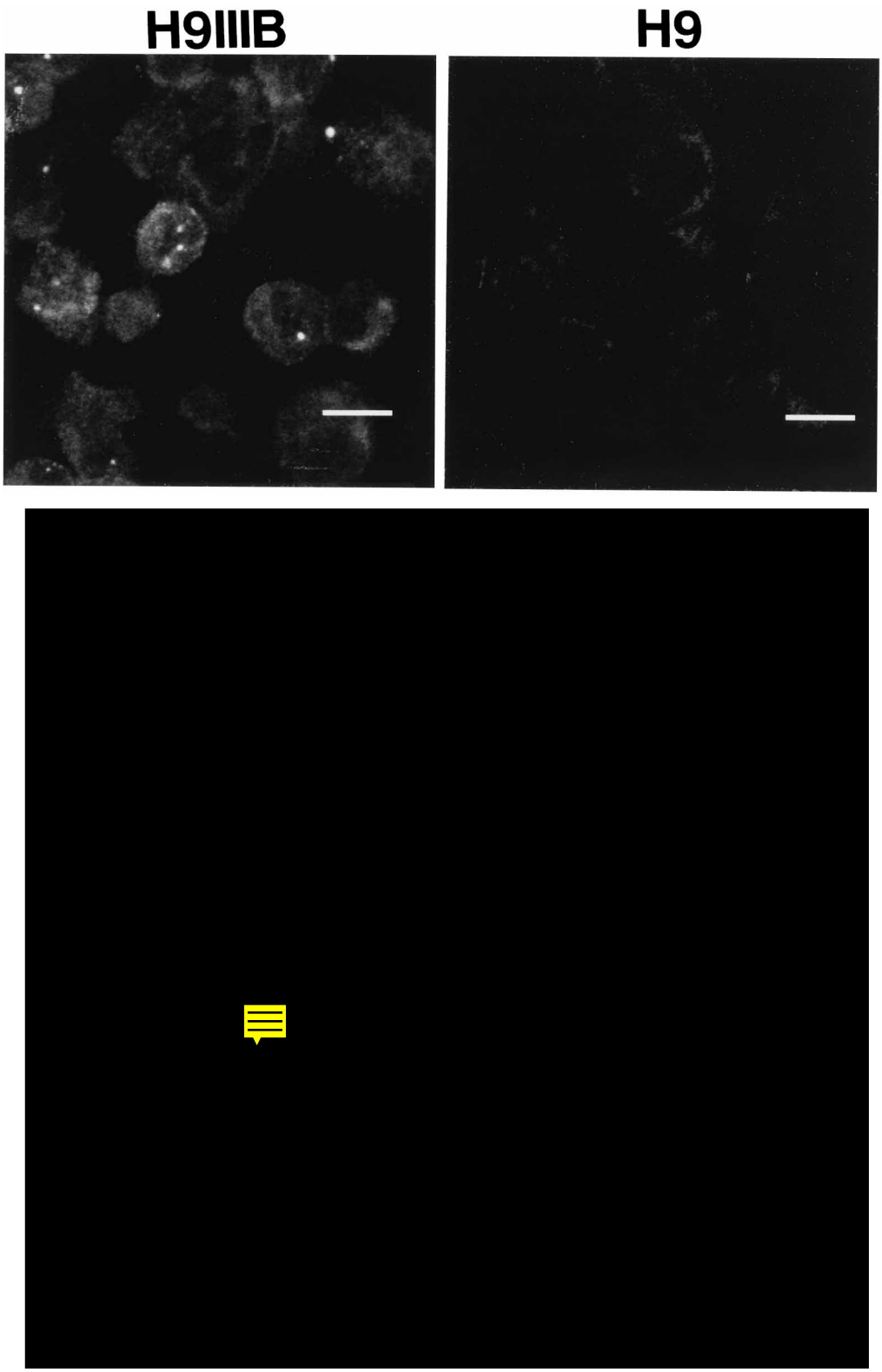

FIG. 4. Detection of HIV unspliced RNA in HIV-infected H9 cells. (Top) H9 cells chronically infected with HIV IIIB or control uninfected H9 cells were cytospun onto microscope slides, fixed, and hybridized in situ to HIV probe B (Fig. 1). Representative cells demonstrating HIV-specific signals were detected in chronically infected but not in uninfected cells. Bar = 10 μm. (Bottom) Detection of unspliced RNA after HIV cell-cell transmission. H9 cells chronically infected with HIV pNL4-3 were mixed with uninfected H9 cells (one infected cell to four uninfected cells) to establish rapid HIV cell-cell transmission. At 21 h after cocultivation, cells were harvested and hybridized in situ (a) using probe B (Fig. 1) and immunostained for SC35 (b). The acquired images were recombined and pseudocolored (RNA is red, SC35 is green) to investigate colocalization (c); the corresponding Nomarski image (d) is included to identify cell-cell fusion. Bar = 10 μm.

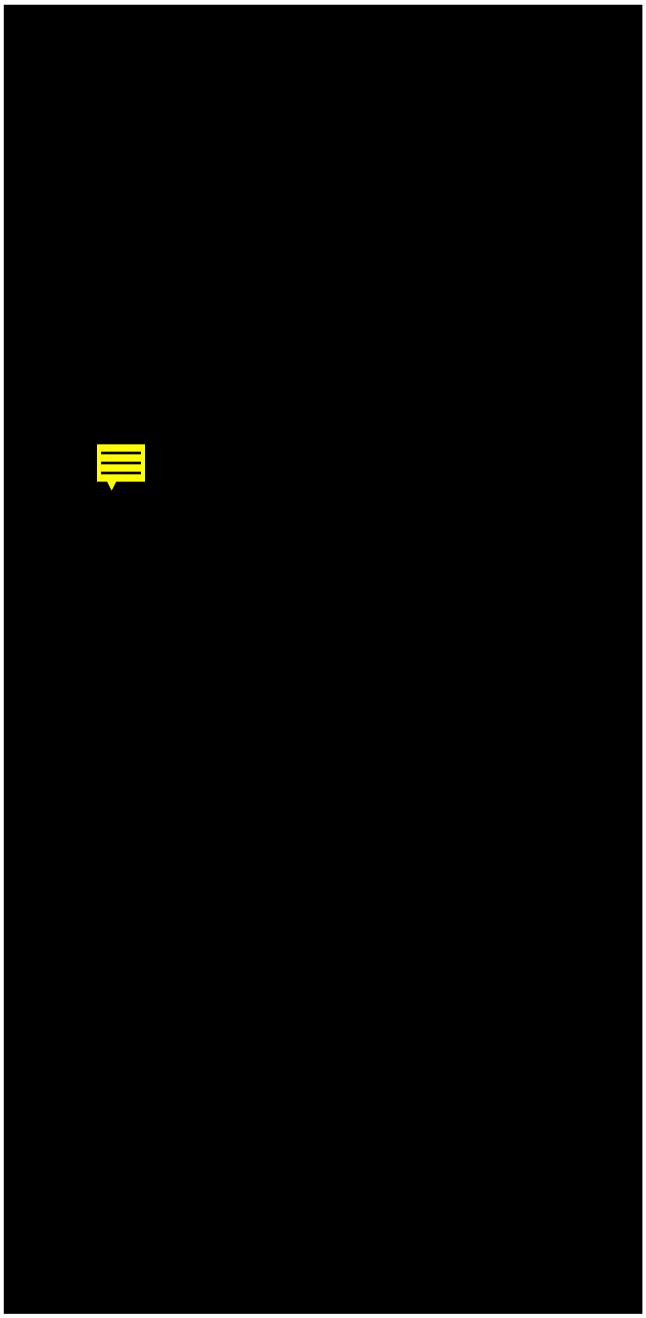

FIG. 5. Comparison of intranuclear distributions of unspliced HIV mRNAs in the presence and absence of *rev* expression. HeLa cells were transfected with *rev*+ (a, c, e, and g) or *rev*− (b, d, f, and h) pNL4-3, and cells were hybridized with ACC probe (a and b) and immunostained for SC35 (c and d). The images acquired were recombined and pseudocolored (RNA is red, SC35 is green) to investigate colocalization (e and f); the corresponding Nomarski image (g and h) is included. *rev* expression allowed RNA expression in the nucleus and cytoplasm (a); overlay of the SC35 immunostaining image (c) onto the in situ hybridization image (a) revealed no significant colocalization (e). In the absence of *rev*, unspliced RNA (b) was confined to the nucleus (compare with the nuclear outline evident in the Nomarski image in panel h). Recombining the RNA image (b) with the corresponding SC35 immunostaining (d) revealed no significant colocalization (f; RNA is red, SC35 is green, colocalization is yellow). Bar = 10 μm.

quantitative assay of Gag protein production revealed only trace amounts of p24 protein (10 pg per 10^6 cells) present in HeLa cells transfected with this construct.

To determine whether the presence of the RRE sequence altered HIV RNA intranuclear localization, a derivative of pCMVgag-2 was constructed containing the RRE sequence 3′ to the *gag* coding sequence (Fig. 6, pCMVgag-2RRE), and FISH-immunofluorescence was performed after transient expression in HeLa cells. As shown in Fig. 8b, addition of RRE to pCMVgag-2 plasmid did not result in marked changes in the distribution of unspliced *gag* RNA; as shown in the composite image in Fig. 8h, no colocalization of the *gag* RNA in situ hybridization signal with the SC35 immunostaining signal was detected (Fig. 8b, RNA; Fig. 8e, SC35; Fig. 8h, overlay). Addition of the RRE alone did not allow *gag* expression in the cytoplasm; no RNA hybridization signal was identified in the cytoplasm of transfected cells (Fig. 8b), and only trace p24 protein production was detected by antigen capture assay (<10 pg of p24 per 10^6 cells). However, *gag* expression from pCMVgag-2RRE was *rev* responsive; cotransfection of *rev*-expressing plasmid with pCMVgag-2RRE resulted in marked cytoplasmic accumulation of unspliced *gag* RNA (Fig. 8c), and a 10-fold increase in p24 protein production was detected (100 pg of p24 per 10^6 cells) compared with that measured in pCMV gag-2-transfected cells. The distribution of unspliced *gag* RNA in the nucleus expressed from pCMVgag-2RRE in the presence of Rev (Fig. 8c) was similar to that of pCMVgag-2RRE alone (Fig. 8b); a composite image (Fig. 8i) of the in situ hybridization signal of unspliced *gag* RNA expressed from pCMVgag-2RRE plus Rev (Fig. 8c) and SC35 immunostaining (Fig. 8f) revealed no colocalization of *gag* RNA with SC35-containing speckles.

To investigate the distribution of the *rev* gene product, we performed additional immunofluorescence studies to identify Rev intranuclearly. Rev immunostaining revealed that the majority of Rev was present in or surrounding the nucleolus (Fig. 9a); Rev signal in the nucleoplasm was generally distributed in a finely granular pattern (Fig. 9a). Costaining with anti-SC35 (Fig. 9b) and overlay of the Rev protein (red) and SC35 protein (green) signal revealed that the majority of Rev protein did not accumulate with SC35 (Fig. 9c), although some association with SC35-containing granules was present, usually near the periphery of granules rather than a precise colocalization (Fig. 9c). In these transient expression studies, the distribution of Rev protein in the nucleoplasm was similar to the distribution of unspliced *gag* RNA. These findings suggest the possibility that a portion of the *rev* gene product is present in the same nuclear compartment as unspliced *gag* RNA.

Features present in the *gag* coding sequence and the RRE direct intranuclear localization. To determine whether *cis*-acting *gag* repressor sequences participated in intranuclear distribution of *gag* RNA, a 719-nt deletion in the *gag* gene was constructed which removes an intragenic *cis*-inhibitory sequence and RNA expression from the resulting plasmid, pCMVgag-2ACC (Fig. 6), was investigated by transient expression in HeLa cells. In contrast to the RNA distribution expressed from pCMVgag-2 (Fig. 10a), unspliced RNA from the *gag* deletion construct was consistently present in the nucleus in large structures (Fig. 10b). The distribution of unspliced RNA expressed from pCMVgag-2ACC (Fig. 10b) was similar to the distribution of SC35-containing nuclear speckles (Fig. 10e). Combining the in situ hybridization image (Fig. 10b) and the SC35 immunostaining image (Fig. 10e) revealed that the large intranuclear collections of *gag* RNA colocalized with SC35-containing nuclear speckles (Fig. 10h, colocalization in yellow). Thus, these data indicate that the removal of *cis* se-

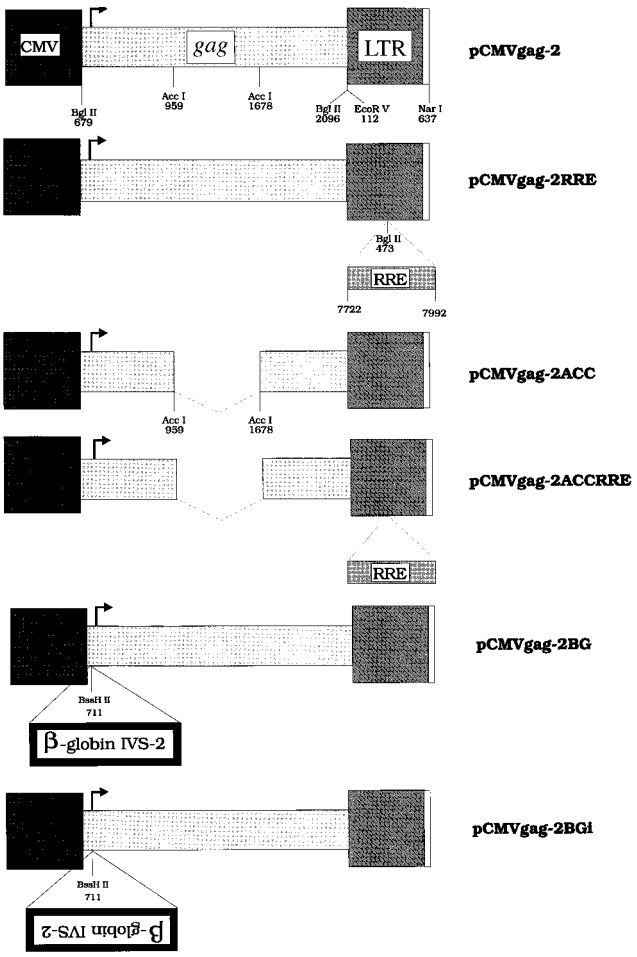

FIG. 6. Schematic of HIV *gag* expression plasmids. *gag* expression plasmids were constructed containing the cytomegalovirus immediate-early promoter directing the HIV *gag* gene and the HIV LTR as a polyadenylation signal (pCMVgag-2) as described in Materials and Methods; a derivative was cloned containing the HIV RRE cloned into a *Bgl*II site 3′ to the *gag* coding sequence (pCMVgag-2RRE). Removal of *cis*-inhibitory sequences by *Acc*I deletion of pCMVgag-2 and pCMVgag-2RRE yielded pCMVgag-2ACC and pCMVgag-2ACCRRE, respectively. pCMVgag-2BG contains the β-globin IVS-2 intron cloned in the sense orientation 5′ to the major splice donor in the *Bss*HII site; pCMVgag-2BGi contains the IVS-2 intron cloned in the inverted orientation.

quences altered the intranuclear accumulation of *gag* RNA. To investigate whether the presence of the RRE sequence affected the intranuclear localization of the deleted *gag* RNA, a plasmid, pCMVgag-2ACCRRE, was constructed containing the intragenic *gag* deletion and the RRE sequence 3′ to the remaining *gag* coding sequence (Fig. 6). In marked contrast to the findings with pCMVgag-2ACC, none of the unspliced RNA expressed from pCMVgag-2ACCRRE was associated with SC35-containing clumps (Fig. 10c). Combining the in situ hybridization image of RNA expressed from pCMVgag-2AC CRRE (Fig. 10c) with the SC35 immunostaining image (Fig. 10f) revealed only random colocalization (Fig. 10i). Coexpression of Rev with pCMVgag-2ACCRRE did not alter *gag* intranuclear RNA localization, and the *gag* RNA appeared similar to unspliced *gag* RNA from pCMVgag-2ACCRRE alone (data not shown). These observations indicate that, like the *cis*-acting intragenic *gag* sequence, the RRE directed intranuclear localization of unspliced *gag* RNA.

Redirection of intranuclear RNA localization correlates with increased Gag expression. The finding that specific HIV *gag* RNA domains redirected intranuclear localization did not permit us to examine the effect of nuclear localization on Gag protein expression because the removal of the *cis*-acting sequence disrupted the *gag* reading frames. As an alternative, we investigated a cellular *cis*-acting sequence which, when placed upstream of the *gag* coding region, redirected *gag* RNA and which permitted *rev*-independent Gag expression. Previously, Hammarskjöld and coworkers (27) determined that the presence in *cis* of the second intervening sequence from rabbit β-globin (IVS-2), permitted *rev*-independent expression of HIV *env*. We therefore investigated whether the presence of the IVS-2 in *cis* redirected *gag* RNA localization and conferred *rev*-independent Gag expression. We constructed recombinant plasmids derived from pCMVgag-2 which contained β-globin IVS-2 in the sense orientation (pCMVgag-2BG) or, as a control, in the inverted, reverse complement orientation (pCMVgag-2BGi, Fig. 6) and then analyzed the intranuclear distribution of unspliced HIV *gag* RNA and the production of Gag protein after transient expression in HeLa cells. Introduction of the β-globin IVS-2 into pCMVgag-2 in the sense orientation (pCMVgag-2BG) resulted in the distribution of RNA into large granules (Fig. 11b). Immunostaining with SC35 antibody (Fig. 11e) and overlay of the RNA hybridization and SC35 immunofluorescence signals (Fig. 11h) revealed marked colocalization (Fig. 11h; *gag* RNA is red, SC35 is green). In contrast, *gag* RNA expressed from pCMVgag-2 (Fig. 11a) or from the control plasmid pCMVgag-2BGi, containing the β-globin IVS-2 in the inverted orientation (Fig. 11c), was present in small granules. Costaining of pCMVgag-2 and pCMVgag-2BGi with SC35 (Fig. 11d and f, respectively) and overlay of the SC35 immunofluorescence and RNA hybridization signals revealed no colocalization between SC35 and *gag* RNA expressed from pCMVgag-2 (Fig. 11g; RNA is red, SC35 is green) or pCMVgag-2BGi (Fig. 11i; RNA is red, SC35 is green). These findings revealed that the presence of the β-globin IVS-2 in *cis* resulted in a marked intranuclear redistribution of unspliced HIV *gag* RNA into SC35-containing granules.

To investigate whether redirecting *gag* RNA into SC35-containing granules was associated with an increase in Gag protein expression, we performed FISH-immunostaining experiments to detect the expression of *gag* RNA and Gag protein (Fig. 12). Gag protein expression was undetectable in cells transfected with pCMVgag-2 (Fig. 12a, *gag* RNA; Fig. 12d, Gag protein immunostaining). In contrast, expression from pCMVgag-2BG containing the β-globin IVS-2 in the sense orientation resulted

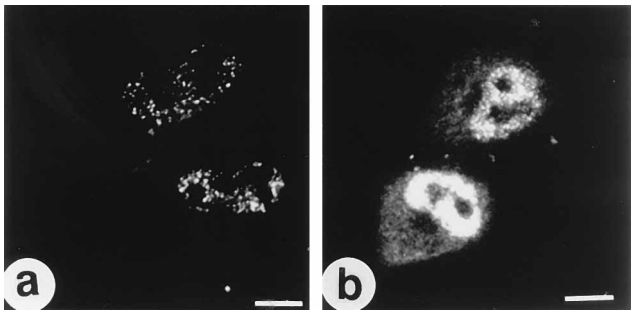

FIG. 7. Identification of unspliced and spliced mRNA expressed from pCMVgag-2. HeLa cells were transfected with pCMVgag-2, and mRNA was detected in situ with biotinylated DNA probe ACC (a) to detect unspliced *gag* RNA or with biotinylated pCMVgag-2 plasmid probe (b) to detect spliced and unspliced RNA. Bar = 10 μm.

FIG. 8. Identification of HIV *gag* RNA from recombinant expression plasmids. HeLa cells transfected with pCMVgag-2 (left column), pCMVgag-2RRE (middle column), or pCMVgag-2RRE and pCMV-Rev (right column) were fixed and hybridized to *gag* probe ACC (a to c) and immunostained with anti-SC35 (d to f). The presence of colocalization was determined by overlaying the RNA and SC35 images (g to h; RNA is red, SC35 is green, colocalization is yellow). The corresponding Nomarski image (j to l) was acquired for each field. Unspliced RNA from pCMVgag-2 (a) contained exclusively nuclear RNA. Combining the in situ hybridization image (a) and the corresponding SC35 image (d) revealed little colocalization (g). The addition of the RRE did not change the pattern of nuclear RNA (b); overlay of the SC35 image (e) yielded no significant colocalization (h). Cotransfection of pCMVgag-2RRE with *rev*-expressing pCMV-Rev resulted in expression of unspliced RNA in the nucleus and cytoplasm (c). Combining the SC35 immunostaining image (f) with the RNA hybridization image (c) revealed little colocalization (i; RNA is red, SC35 is green). Bar = 10 μm.

in Gag protein expression (Fig. 12b, unspliced *gag* RNA; Fig. 12e, Gag protein immunostaining). Quantitative p24 antigen determinations revealed a sevenfold increase in p24 production in pCMVgag-2BG-transfected cells (70 pg of p24 per 10^6 cells) compared with that expressed in pCMVgag-2 (10 pg of p24 per 10^6 cells). Gag protein expression was undetectable from cells expressing the control plasmid pCMVgag-2BGi (Fig. 12c, unspliced *gag* RNA; Fig. 12f, Gag protein immunostaining). Quantitative p24 determinations confirmed that only trace amounts (10 pg of p24 per 10^6 cells) of Gag protein were expressed in pCMVgag-2BGi-transfected cells. The finding that the presence of the β-globin IVS-2 permitted *rev*-independent Gag protein expression and redistributed *gag* RNA similar to that obtained for *rev*-independent *tat* transcripts (compare Fig. 11b and 12b with Fig. 3c) suggests that redirecting nuclear RNA localization may affect gene expression.

DISCUSSION

HIV-1 expression proceeds by a regulated program of alternative splicing of full-length 9.2-kb primary transcripts into three different size classes of mRNA. Unspliced 9.2-kb mRNA and singly spliced 4.0-kb mRNA species require the binding of the HIV gene product Rev to the RRE for cytoplasmic expression, whereas multiply spliced 1.8-kb transcripts are expressed in the presence or absence of *rev*. The in situ hybridization findings presented here reveal that *rev*-dependent and *rev*-independent RNA transcripts accumulate in distinct intranuclear sites, demonstrating that the alternatively spliced products of HIV are in turn alternatively localized within the nucleus. *cis* sequences were responsible for intranuclear localization, and the portion of the *gag* gene we investigated contains inhibitory regions which are capable of repressing their own expression and which are rescued by the RRE in *cis* and by the *rev* gene product in *trans* (12, 42, 65). Inhibitory sequences have been suggested to function in regulating HIV RNA processing by several potential mechanisms, including reducing the half-life of RNA, retaining RNA in the nucleus, and preventing translation of RNA in the cytoplasm (12, 42, 64, 65). In the experiments reported here, expression of *gag* RNA from *rev*-defective pNL4-3 or from the pCMVgag-2 plasmid yielded nuclear RNA containing inhibitory sequences present exclusively in the nucleus, consistent with previous biochemical fractionation studies of transient expression of recombinant chloramphenicol acetyltransferase reporter plasmids containing *gag/pol* inhibitory sequences (42). Our finding that the inhibitory regions may direct *gag* RNA localization suggests that *cis* repression may be accomplished through nuclear compartmentalization. Sequence-dependent RNA localization has been proposed (38) as a mechanism by which cellular gene expression may be regulated in the nucleus and compartmentalized in the cytoplasm (34, 41, 60). Our findings suggest that, in addition to defining intron/exon boundaries and polyadenyation and methylation sites, HIV pre-mRNA transcripts contain signals which determine its nuclear accumulation site and may affect its processing and expression.

The *rev* gene product is an RNA binding protein capable of shuttling between the nucleus and the cytoplasm (33, 49, 59); the reported models for the mechanism of Rev function include: (i) improving the efficiency of nucleocytoplasmic transport (19, 25, 26, 44); (ii) influencing splice site utilization (39); (iii) facilitating dissociation from splicing complexes (9, 35, 36); (iv) stabilizing unspliced RNA (20, 66); and (v) activating HIV mRNA translation in certain cell types (5, 14, 59). Rev protein has been detected predominantly in the nucleolus (13, 57) and in the nucleoplasm (33, 40), associated, in part, with SC35-containing granules. In our experiments, Rev was present predominantly in the nucleoli and in small SC35-independent granules in the nucleoplasm (Fig. 9). Thus, a portion

FIG. 9. Independent distributions of Rev protein and SC35. HeLa cells transfected with pCMVgag-2RRE and pCMVRev were immunostained for Rev (a) and SC35 (b); overlay of the corresponding images (c; Rev is red, SC35 is green, colocalization is yellow) revealed distinct distributions, although some Rev protein associated with the periphery of SC35-containing granules. Bar = 10 μm.

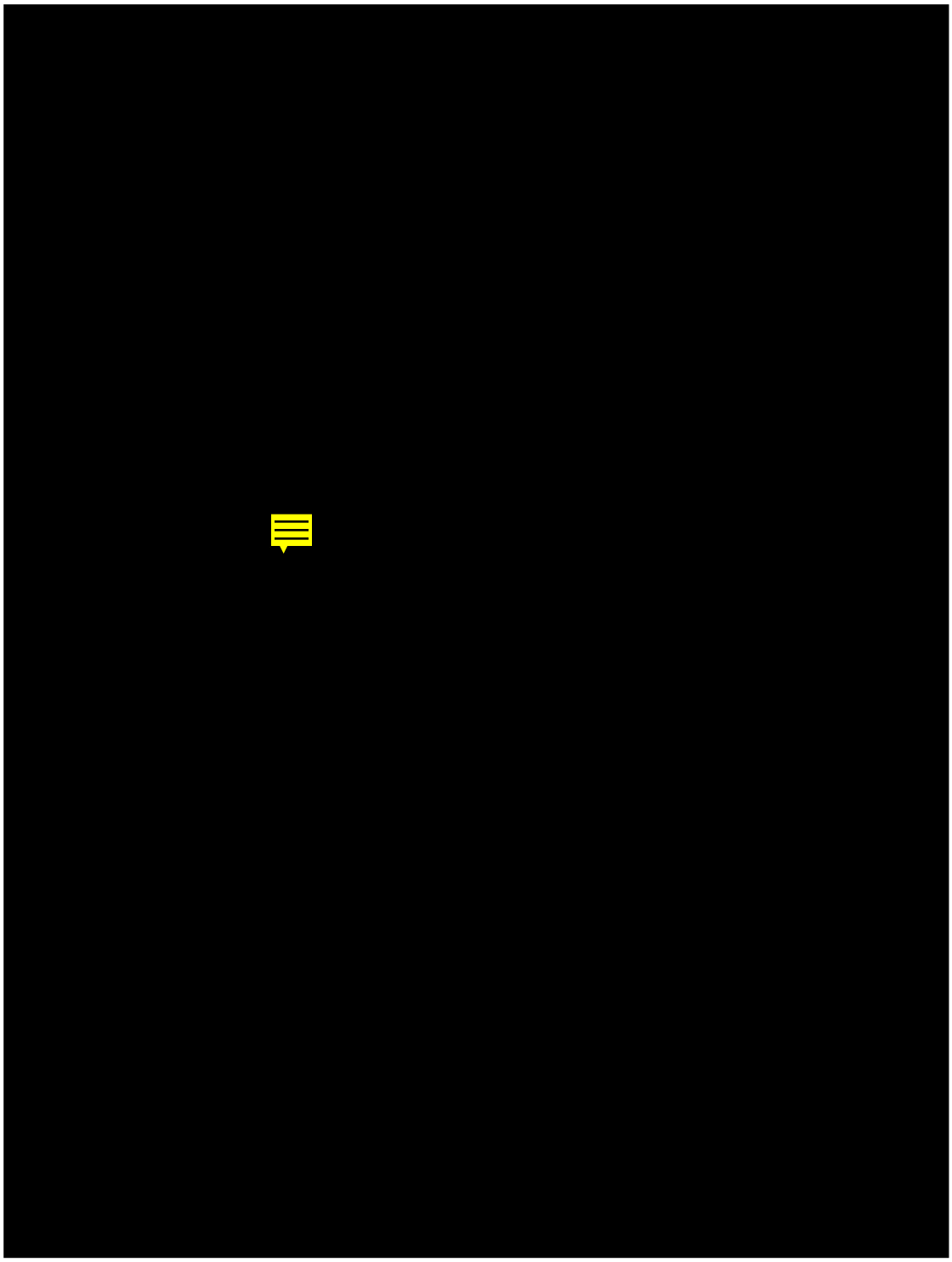

FIG. 10. *cis*-acting sequences direct RNA localization. HeLa cells transfected with pCMVgag-2 (left column), pCMVgag-2ACC (middle column), or pCMVgag-2ACCRRE (right column) were hybridized to probe D, and confocal images of RNA hybridization (a to c) and SC35 immunostaining (d to f) were acquired and recombined (g to h) to determine colocalization. Nomarski images (j to l) are included for comparison. Cells transfected with pCMVgag-2 expressed RNA exclusively in the nucleus (a); overlay of SC35-immunostaining image (d) revealed no colocalization (g; RNA is red, SC35 is green). Cells transfected with pCMVgag-2ACC, containing a deletion of *cis*-inhibitory sequences, contained RNA in large accumulations (b); combining the RNA image with the corresponding SC35 immunostaining image (e) revealed marked colocalization (h; RNA is red, SC35 is green, colocalization is yellow). Expression of unspliced RNA from pCMVgag-2ACCRRE, containing the deletion in the *gag* inhibitory sequence and the addition of the RRE (Fig. 6), resulted in RNA present in fine granules (c); overlay with the corresponding SC35 image (f) revealed that these transcripts no longer colocalized with SC35 (i; RNA is red, SC35 is green). Bar = 10 μm.

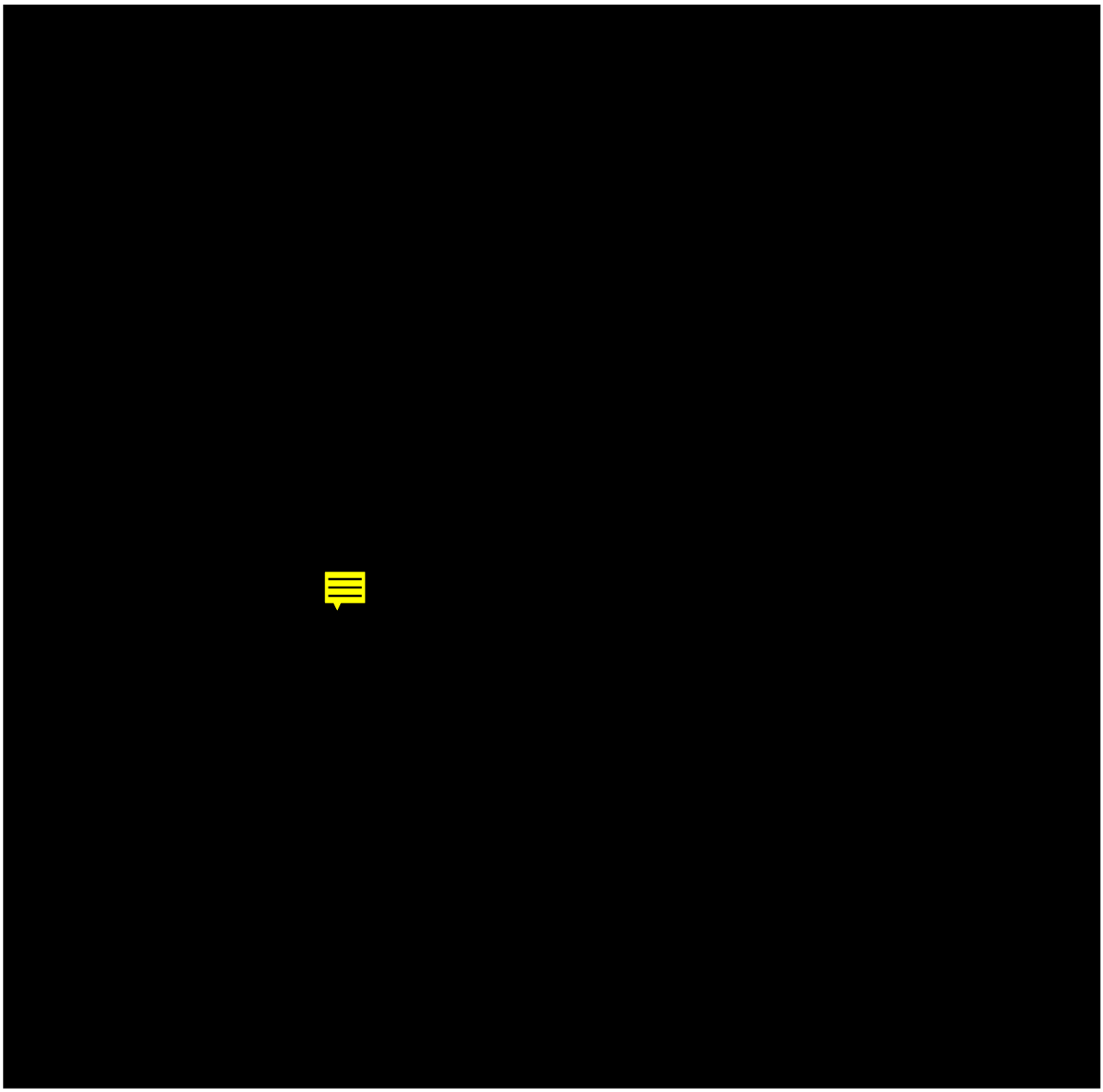

FIG. 11. Redirection of *gag* RNA by β-globin intron in *cis*. HeLa cells were transfected with pCMVgag-2 (a, d, and g), pCMVgag-2BG (b, e, and h) containing the β-globin intron upstream of the major splice donor in the sense orientation, or pCMVgag-2BGi containing IVS-2 in the inverse complement orientation (Fig. 6). In situ hybridization using probe ACC (a, b, and c) to detect unspliced RNA revealed that the presence of the β-globin intron redirected HIV unspliced RNA into large granules (b) compared with the distribution of unspliced RNA in small granules expressed from pCMVgag-2 (a) or pCMVgag-2BGi (c). Immunostaining for SC35 (d, e, and f) and combining the corresponding in situ and immunofluorescence images (g, h, and i; RNA is red, SC35 is green) revealed that *gag* RNA expressed from pCMVgag-2BG associated with SC35-containing granules (h), whereas no colocalization was detected between SC35 and unspliced RNA expressed from pCMVgag-2 (g) or pCMVgag-2BGi (i). Bar = 10 μm.

of Rev is present in the same compartment in which we detected unspliced and singly spliced, *rev*-dependent RNA transcripts. It is possible that *cis*-acting sequences direct RNA into the nucleoplasm where Rev is available to bind the RRE and shuttle the RNA to the cytoplasm.

In our experiments, both *gag* inhibitory sequences and the RRE contained RNA localization activity (Fig. 10a, b, and c); since the RRE sequence itself may contain an inhibitory sequence (6), it is possible that we detected the effect of an RRE-associated inhibitory sequence on the localization of pCMVgag-2ACCRRE RNA (Fig. 10c). We have not yet investigated the nuclear localization activity of other *cis*-repressor sequences in *env* to determine whether they all contain a similar localization activity. Interestingly, Lawrence and coworkers (37) identified unspliced HIV RNA, containing *tat* exons and a portion of the *env* sequence, including the RRE

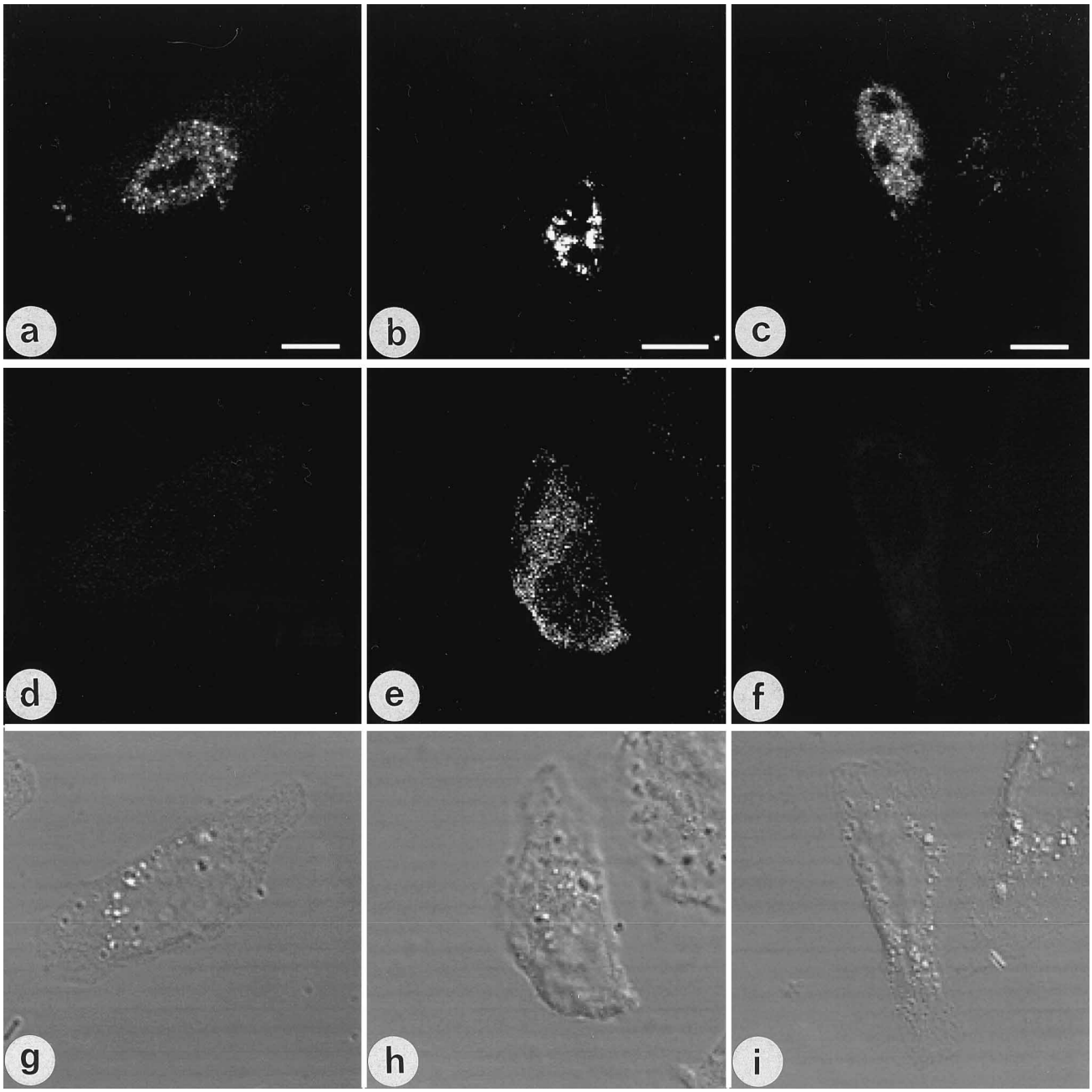

FIG. 12. Rev-independent expression of *gag* RNA containing β-globin IVS-2 in *cis*. HeLa cells were transfected with pCMVgag-2 (a, d, and g), pCMVgag-2BG (b, e, and h), or pCMVgag-2BGi (c, f, and i); unspliced RNA was detected by in situ hybridization using probe E (a, b, and c), and p24 was detected using fluorescein isothiocyanate-conjugated anti-p24 monoclonal antibody (d, e, and f). Cells transfected with pCMVgag-2 (a) or pCMVgag-2BGi (c) contained unspliced *gag* RNA present in small granules (a), but no Gag protein was detected by p24 immunostaining (pCMVgag-2 and pCMVgag-2BGi, panels d and f, respectively). In contrast, cells transfected with pCMVgag-2BG contained unspliced *gag* RNA in large granules (b) and p24 protein expressed in the cytoplasm (e). Corresponding Nomarski images are presented (g, h, and i). Bar = 10 μm.

and repressor sequences, in both a stippled pattern and a large patchy distribution in nuclei of COS-7 cells, and these monkey cells contained unspliced HIV RNA in the cytoplasm in the absence of *rev*. It is likely that the differences in nuclear/cytoplasmic distribution of RNA in COS and HeLa cells detected by in situ and biochemical techniques represent cell-type and/or species-specific differences, but it will be of interest to determine whether transport in COS cells may take place by both a *rev*-dependent and a *rev*-independent pathway.

Ultrastructural and biochemical studies of gene expression suggest a dynamic relationship between the physical organization of the nucleus and the functional events of pre-mRNA processing. Recent visualization of RNA splicing occurring along transcript "tracks" extending from transcription sites to the nuclear periphery suggests that RNA processing is directly coupled to RNA synthesis (8, 30, 72–74). Although tracks may occur close to, or coincident with, SC35-containing nuclear speckles, many tracks are clearly distinct from any large gran-

ules and the role of nuclear speckles is uncertain. Since splicing factors have been reported to accumulate within speckles (7, 67, 68), several investigators have suggested that nuclear speckles may represent repositories from which splicing factors are recruited (30, 31, 75). Others have detected marked reorganization of nuclear speckles upon cell differentiation and dissolution of speckles upon transcriptional arrest, suggesting a more dynamic function for SC35-containing granules (3, 50, 54, 73). Huang and colleagues (30), noting the substantial portion of nuclear RNA associated with SC35-containing structures, have suggested that speckle-associated RNA may play a role in RNA processing itself. It is also possible that SC35-containing granules have functions in addition to storing splicing factors; for example, our study revealed that *tat* RNA was concentrated in some SC35-containing granules, whereas other SC35-containing structures in the same nucleus had little or no *tat* RNA. Recently, Luznik and coworkers (40) described changes in the appearance of SC35-containing granules after HIV infection. It will be of interest to determine whether such changes occur in all SC35-containing granules or in those containing *tat* RNA. In addition, we are investigating whether association of *tat* RNA with nuclear speckles requires specific sequences within multiply spliced HIV RNAs or occurs because of the absence of inhibitory-type signals.

The functional consequences of redirecting RNA to distinct intranuclear locations remain uncertain. Our experiments identified that a cellular sequence, IVS-2 from rabbit β-globin, may also direct RNA, indicating that RNA localization sequences are not restricted to HIV and that redirecting intranuclear localization is associated with differences in gene expression. In our studies, the presence of the β-globin intron resulted in *rev*-independent expression, similar to the previously reported effect of the β-globin intron on *env* expression. The intranuclear distribution of *rev*-independent *gag* RNA expressed from pCMVgag-2BG was similar to the distribution of *rev*-independent *tat* RNA. We speculate that the model proposed by Hammarskjöld and coworkers (27) to explain *rev*-independent *env* expression may apply to *rev*-independent *gag* expression as well and that it includes redirecting RNA into a distinct pathway, reflected in our experiments as a marked change in intranuclear accumulation into SC35-containing granules.

The mechanism of how intragenic sequences affect nuclear RNA distribution presumably involves interactions with nuclear factors. Several novel nuclear proteins binding the RRE have been identified (56, 71) whose precise functions in HIV RNA processing remain to be elucidated. A specific interaction between a *cis*-repressor sequence in *pol* and an abundant nuclear factor, heterogeneous nuclear ribonucleoprotein C (hnRNP-C), has been reported (55). hnRNP-C binds preferentially to U-rich sequences along RNA transcripts and may influence 3′ splice site selection (10, 18, 23). HIV *gag*/*pol* sequences contain AU-rich regions, which may account for hnRNP-C binding; interestingly, Schwartz and coworkers (64) demonstrated that interrupting multiple U-rich sequences results in loss of the inhibitory effect of *gag* sequences and renders them *rev* independent. It is conceivable that binding of nuclear factors such as hnRNP-C to intragenic sequences may be required for *cis* repression, but little is known about the interactions of nuclear factors and RNA processing in the context of the intact nucleus. By combining in situ localization and RNA binding studies to characterize functional and spatial relationships between inhibitory sequences and nuclear factors, we hope to identify the events in RNA processing critical to HIV replication, which may serve as potential sites for therapeutic intervention.

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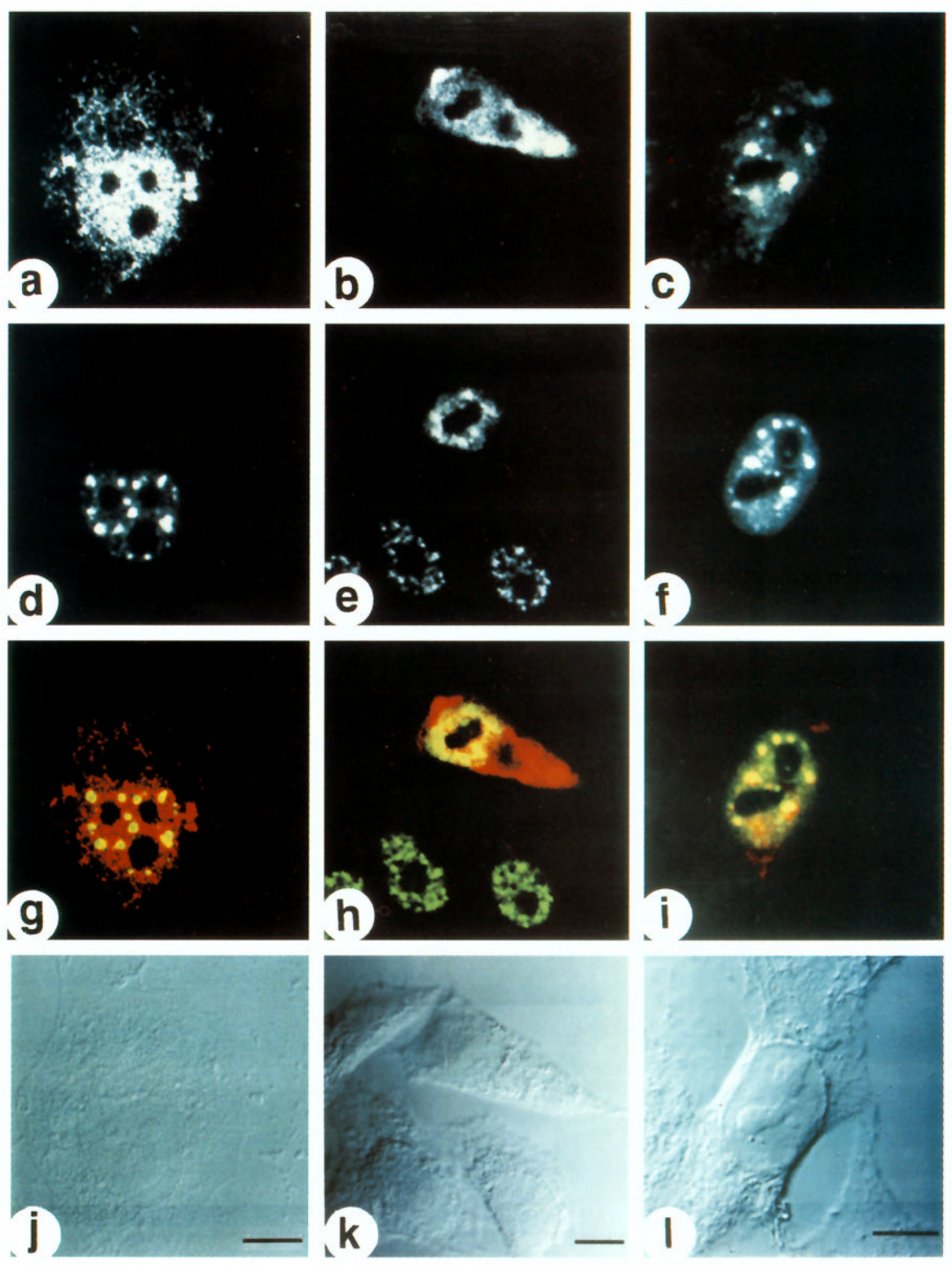
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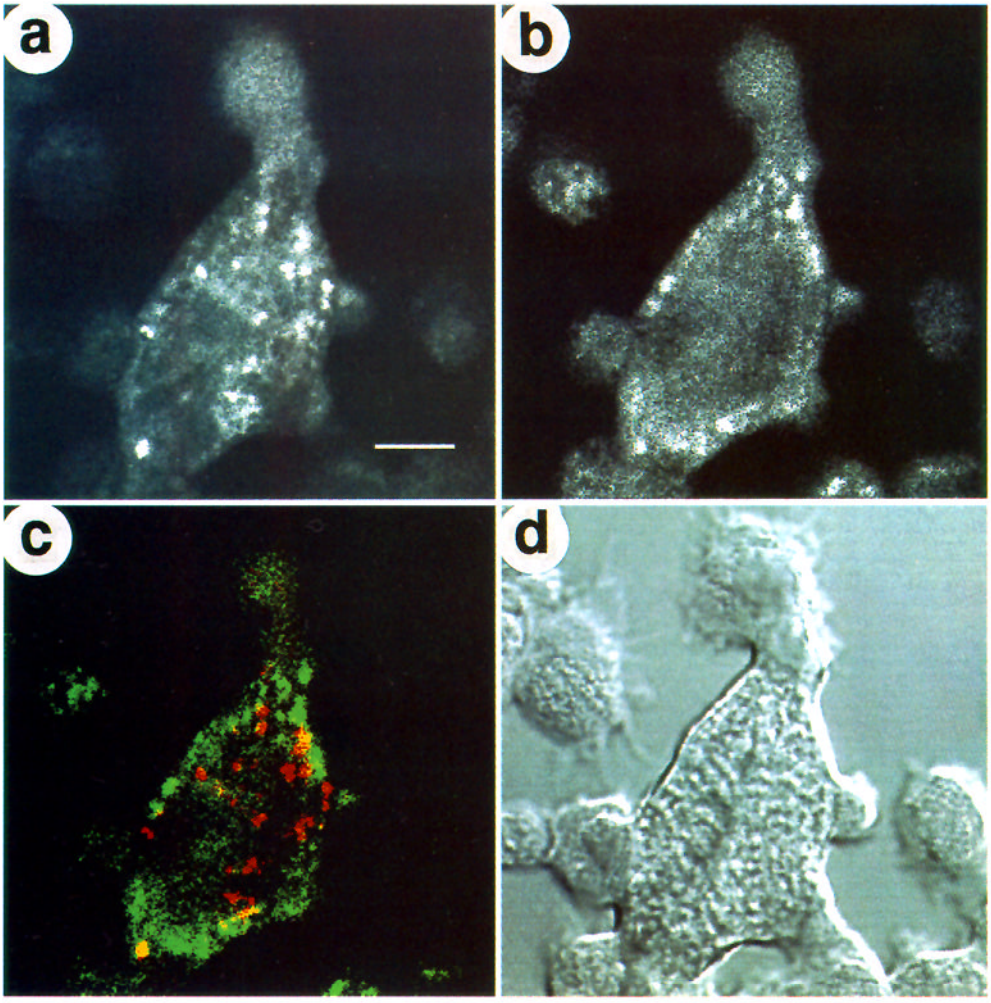
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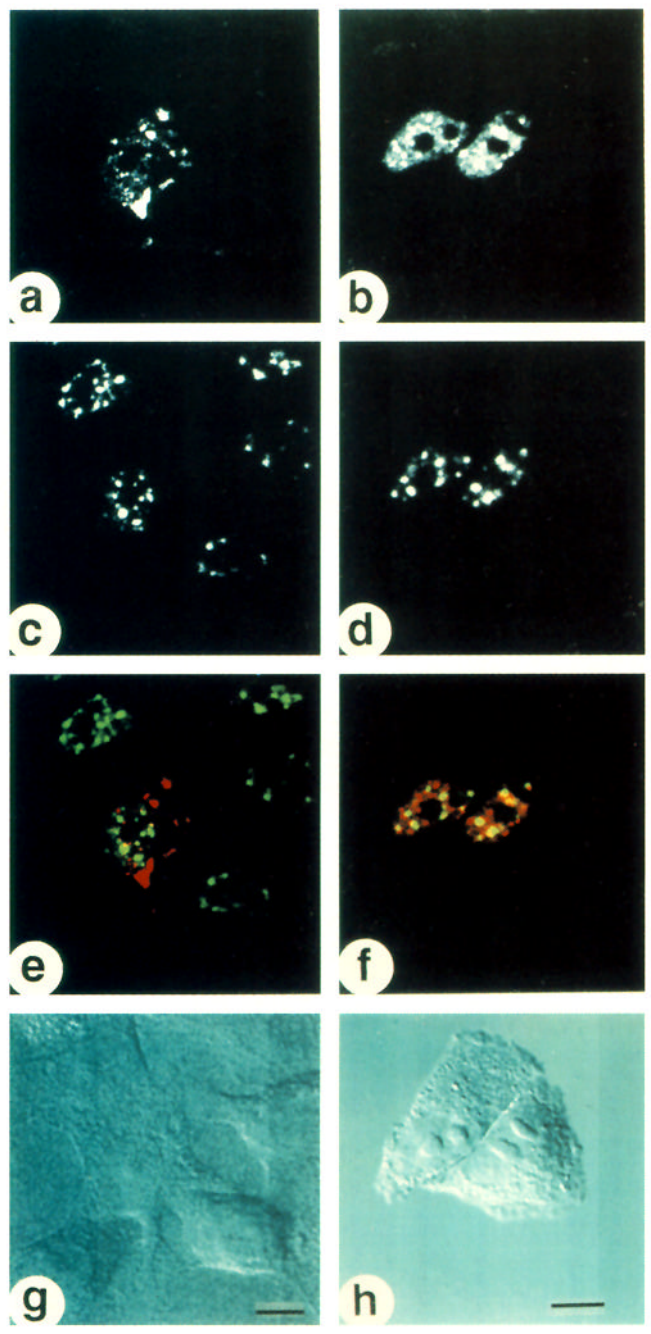
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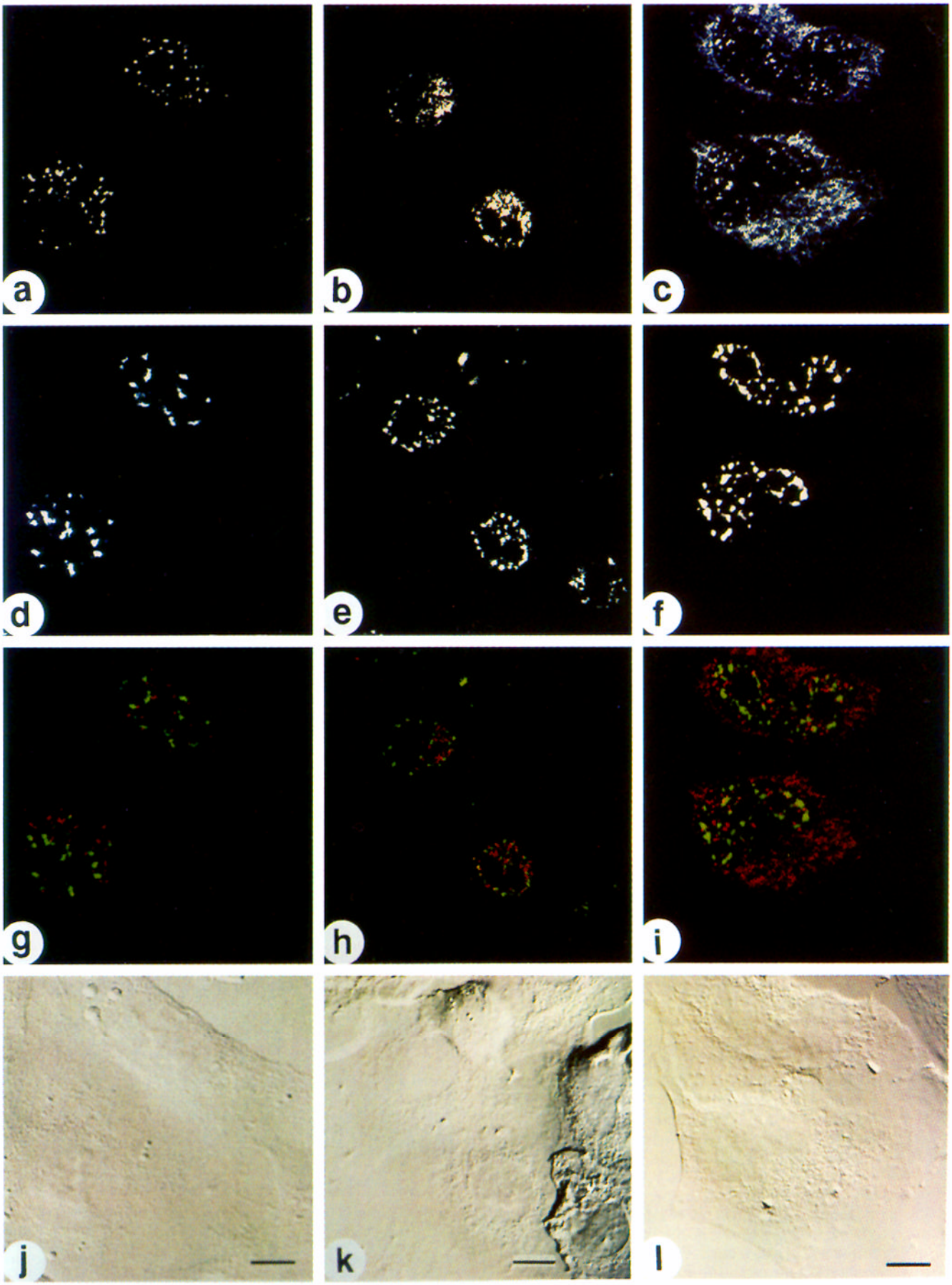
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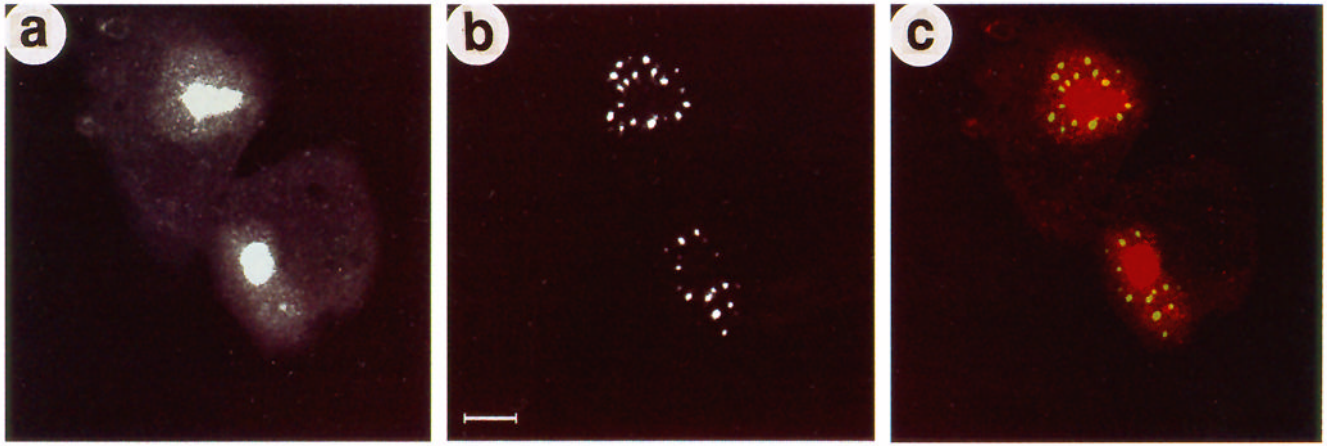
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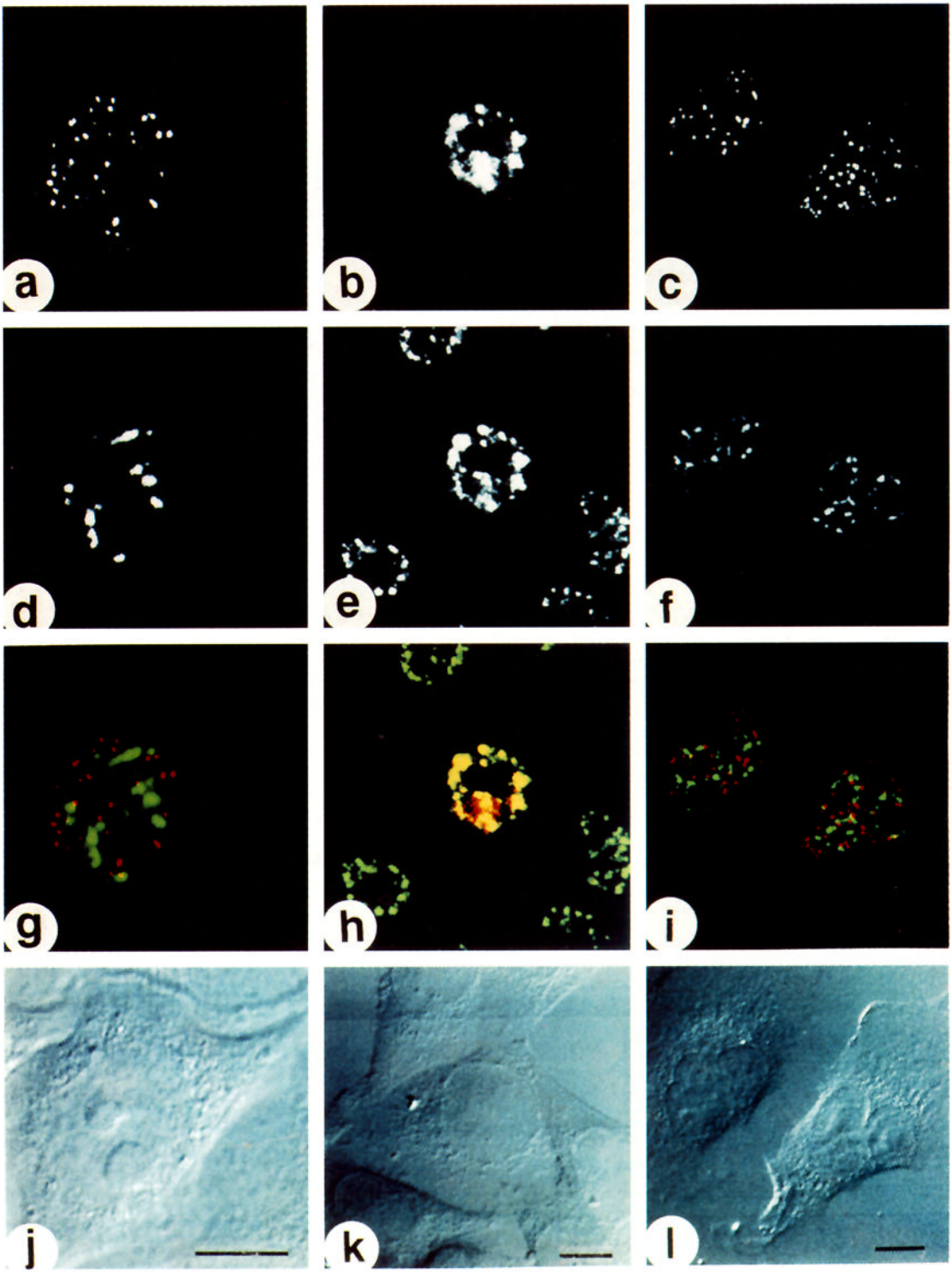
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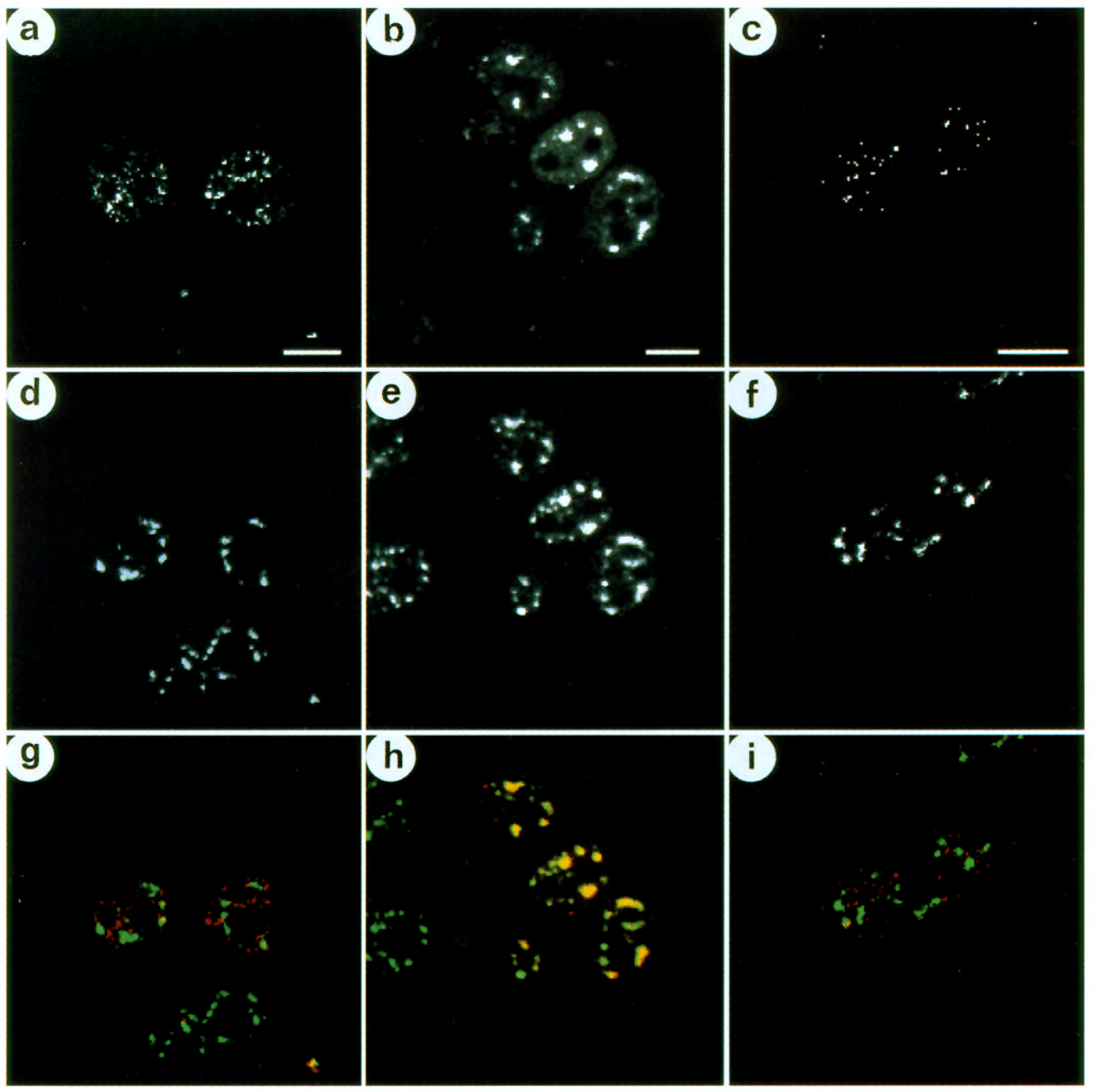
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