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Herpes Simplex Virus Type 1 Protein IE63 Affects the Nuclear Export of Virus Intron-Containing Transcripts

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Using in situ hybridization labelling methods, we have determined that the herpes simplex virus type 1 immediate-early protein IE63 (ICP27) affects the cellular localization of virus transcripts. Intronless transcripts from the IE63, UL38, and UL44 genes are rapidly exported to and accumulate in the cytoplasm throughout infection, in either the presence or absence of IE63 expression. The intron-containing transcripts from the IE110 and UL15 genes, while initially cytoplasmic, are increasingly retained in the nucleus in distinct clumps as infection proceeds, and the clumps colocalize with the redistributed small nuclear ribonucleoprotein particles. Infections with the IE63 mutant virus 27-lacZ demonstrated that in the absence of IE63 expression, nuclear retention of intron-containing transcripts was lost. The nuclear retention of UL15 transcripts, which demonstrated both nuclear and cytoplasmic label, was not as pronounced as that of the IE110 transcripts, and we propose that this is due to the late expression of UL15. Infections with the mutant virus 110C1, in which both introns of IE110 have been precisely removed (R. D. Everett, J. Gen. Virol. 72:651–659, 1991), demonstrated IE110 transcripts in both the nucleus and the cytoplasm; thus, exon definition sequences which regulate viral RNA transport are present in the IE110 transcript. By in situ hybridization a stable population of polyadenylated RNAs was found to accumulate in the nucleus in spots, most of which were separate from the small nuclear ribonucleoprotein particle clumps. The IE63 protein has an involvement, either direct or indirect, in the regulation of nucleocytoplasmic transport of viral transcripts, a function which contrasts with the recently proposed role of herpes simplex virus type 1 Us11 in promoting the nuclear export of partially spliced or unspliced transcripts (J.-J. Diaz, M. Duc Dodon, N. Schaerer-Uthurraly, D. Simonin, K. Kindbeiter, L. Gazzolo, and J.-J. Madjar, Nature [London] 379:273–277, 1996), the significance of which is discussed.

Primary transcripts of genes transcribed by RNA polymerase II generally are processed by 5′ capping, splicing, and 3′ processing before transportation to the cytoplasm (reviewed in references 35, 36, and 71). Direct labelling methods have demonstrated that splicing occurs at sites of transcription (80); however, polyadenylated unspliced intermediates can be detected both in vivo and in vitro (53). Splicing of 5′-proximal introns can precede polyadenylation of large transcripts (39), and Liu and Mertz (40) have demonstrated that poly(A) site selection, but not necessarily cleavage, precedes excision of the 3′-terminal intron in vivo; the temporal order in which these RNA processing functions occur is variable. The 5′ cap and poly(A) tail are believed to act as RNA transport signals (13), whereas the presence of introns inhibits transport to the cytoplasm as splicing factors are recruited to intron-containing transcripts which are then retained in the nucleus until they are spliced and/or actively transported to the cytoplasm (2).

A number of factors govern the nucleocytoplasmic transport of mRNA, although this process is still poorly understood. Nuclear export occurs in two stages: (i) transport from the gene to the nuclear periphery and (ii) translocation across the nuclear membrane (13). The first step involves ribonucleoproteins (RNPs) (45), which bind RNA cotranscriptionally (51, 57). The mechanism by which a transcript passes through the nuclear pore is thought to involve RNP interactions; binding of heterogeneous nuclear RNP L correlates with efficient mRNA processing and transport, and heterogeneous nuclear RNP L facilitates binding of other heterogeneous nuclear RNPs to the RNA (41).

Regulation of nucleocytoplasmic transport has been identified in a number of viral systems. The human immunodeficiency virus (HIV) type 1 protein Rev together with a cellular factor, Rab, induces the cytoplasmic appearance of unspliced and partially spliced virus transcripts via a direct and sequence-specific interaction with the Rev response element, which is contained within all intron-containing HIV transcripts (11, 18); a similar mechanism has been identified for the human T-cell leukemia virus (HTLV) type 1 Rex protein (3). In contrast to Rev and Rex, the influenza virus NS1 protein has an inhibitory effect on the transport of spliced polyadenylated RNAs and inhibits splicing (19, 59, 60). The adenovirus E1B protein enhances the relocation of viral mRNAs to particular subnuclear compartments and facilitates their export to the cytoplasm (38, 55). The herpes simplex virus type 1 (HSV-1) Us11 late protein has been implicated as having effects similar to those of Rev and Rex, as it can functionally substitute for their functions in vitro (12). We show here that the HSV-1 IE63 protein acts to inhibit nuclear export of intron-containing transcripts; whether Us11 acts to promote export of unspliced transcripts in HSV-infected cells, in which case IE63 and Us11 would be in apparent competition, remains to be seen.

HSV-1 is a nuclear-replicating DNA virus (64) the genes of which are expressed in a temporal cascade (9, 30). Immediate-early (IE) regulatory genes are expressed first; these stimulate early gene expression, which provides many of the functions required for viral DNA synthesis; and finally the late virus genes, which encode mainly structural proteins, are expressed (64, 77). Of the more than 70 HSV-1 genes, only 4 of those expressed during lytic infection contain introns (21). Thus, the virus has only a very limited requirement for the cell's splicing

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machinery; however, lytically expressed viral transcripts are capped and polyadenylated. Of the four spliced transcripts, three (IE110, IE68, and IE12) are expressed from IE times postinfection, and the fourth (UL15) is expressed from late times (10). Another late gene, UL44, has been associated with splicing, as it is apparently located within an intron and its transcripts are produced only in the absence of splicing (21).

HSV-1 infection represses host protein synthesis (17, 29), and this is initially accomplished by the virion host shutoff function, the action of an incoming viral tegument protein (37, 61, 72), which acts by induction of an RNase activity which destabilizes viral and cellular mRNAs and disrupts polyribosomes (34, 37, 75). A second stage of host shutoff, which requires virus gene expression, further reduces the levels of host protein synthesis (16), and the IE63 protein was required for efficient reduction of host protein synthesis (65), implicating this protein as a strong candidate for the second host shutoff function.

An inhibition of splicing has been identified following HSV infection. Schroder et al. (69) found that splicing of β-tubulin RNA was reduced, and Hardwicke and Sandri-Goldin (24) demonstrated a decrease in the levels of three spliced cellular RNAs which was not due to effects on transcription or RNA stability. More recently, Hardy and Sandri-Goldin (26), using an IE63 mutant virus, 27-lacZ, demonstrated that IE63 was required for inhibition of splicing in vitro, and Hibbard and Sandri-Goldin (28) found a reduction in cellular mRNA levels and nuclear accumulation of pre-mRNAs which required IE63 expression. Inhibition of splicing resulted in the nuclear export of some unspliced transcripts (26), suggesting that regulation of RNA export was also affected.

The IE63 (ICP27) protein is a nuclear phosphoprotein and is one of only two IE functions essential for lytic virus replication (62). IE63 plays a key role in the switch from early to late gene expression at the posttranscriptional level (46, 48, 49, 62, 65, 68), has both positive and negative effects on gene expression (25, 50, 70, 73), and functionally interacts with two other IE proteins, IE175 and IE110 (70, 81). IE63 affects the phosphorylation states of a number of proteins (one of which is thought to be the U1 small nuclear RNP [snRNP]) (66), binds RNA directly (32), and acts to stabilize the 3′ ends of normally labile mRNAs (6). Further, IE63 has been shown to be both necessary and sufficient to cause a redistribution of the cellular splicing snRNPs from a diffuse speckled pattern within the nucleus to a highly punctate distribution (56); this redistribution effect was originally observed by Martin et al. (44). Furthermore, we showed that IE63 colocalizes with the snRNPs (56), and more recently, Sandri-Goldin and Hibbard (66) have shown this interaction by coimmunoprecipitation studies. The distribution of the polyadenylation factors remains largely unaffected during HSV infection (48).

Disruption of snRNP organization can be observed under a number of conditions; for example, inhibition of RNA polymerase II transcription by α-amanitin (7), inhibition of splicing by using antisense RNA molecules or antibodies (54), and heat shock treatments (4) cause snRNPs to cycle into interchromatin granule storage sites. Infections with adenovirus (5, 33, 58) and influenza virus (20) also cause a redistribution of the snRNPs. Adenovirus RNAs are extensively spliced, and the splicing factors are recruited into virus transcription and processing sites; in contrast, influenza virus has little requirement for splicing and inhibits cellular splicing, indicating that the redistributed snRNPs in influenza virus-infected cells are inactive aggregates (20).

IE63 can inhibit splicing and can interact and colocalize with the snRNPs, which suggests that this protein inhibits splicing by removing snRNPs from sites of transcription and polyadenylation. Although splicing is largely inhibited by HSV-1 infection, there is evidence for a residual amount of ongoing splicing, as the few intron-containing viral transcripts and some cellular genes are processed. Presumably this residual splicing could be carried out by the remaining snRNPs diffusely distributed throughout the nucleoplasm.

Previous studies which have examined the effects of HSV-1 on splicing have been based largely on in vitro assays and transient-expression systems. In this study we have directly examined the intracellular localization of spliced and unspliced viral RNAs by using in situ hybridization experiments over a time course of HSV-1 infection. The IE and late intron-containing transcripts studied (IE110 and UL15) were increasingly retained in the nucleus in distinct clumps as infection proceeded, whereas intronless IE and late transcripts (IE63, UL38, and UL44) were detected predominantly in the cytoplasm. Double-labelling experiments have demonstrated that these intron-containing transcripts colocalize with the redistributed snRNP clumps. IE63 is required for the nuclear retention of intron-containing transcripts, since in its absence, all transcripts were predominantly cytoplasmic. Cells infected with an IE110 mutant virus in which both introns have been precisely removed (14) demonstrated a localization of IE110 transcripts in both the nucleus and cytoplasm, showing more nuclear label than would be expected for an intronless transcript but more cytoplasmic label than seen with intron-containing transcripts. This suggests the presence of exon definition sequences in the IE110 transcript by which splicing signals are recognized (53, 80). Interestingly, the UL15 late transcript demonstrated a similar intermediate phenotype with both nuclear and cytoplasmic label, and we believe that this is a reflection of the time postinfection of its expression. We have utilized poly(dT) in situ hybridization to locate polyadenylated RNAs and found poly(A)$^+$ RNA in the cytoplasm and in distinct nuclear spots at later times postinfection. However, only a small proportion of these distinct spots colocalized with the redistributed snRNPs, suggesting the presence of either a stable population of poly(A)$^+$ RNA which is retained in the nucleus but whose function is unknown (8, 31) or transient storage forms of incompletely or abnormally processed pre-mRNA (76).

MATERIALS AND METHODS

Cell culture and viruses. HeLa cells were grown as monolayers in Dulbecco's modified minimal essential medium supplemented with 5% newborn calf serum and 5% fetal calf serum. Vero 2-2 cells (73) were maintained in Glasgow minimal essential medium supplemented with 5% newborn calf serum and 5% fetal calf serum. BHK cells were grown in Glasgow minimal essential medium supplemented with 10% newborn calf serum. All cells were grown at 37°C in an atmosphere of 5% CO_2.

Stocks of wild-type HSV-1 (strain 17+) and the HSV-1 mutant 110C1, a gift from R. Everett, in which both introns of IE110 have been precisely removed (14), were grown at 37°C on BHK monolayers. Virus 27-lacZ, a gift from R. Sandri-Goldin, in which the IE63 gene is inactivated by insertion of a *lacZ* cassette, was grown on the complementing Vero 2-2 cell line (73).

HSV-1 infection of cell monolayers. All cells were grown as monolayers on sterile glass coverslips previously treated with 10% poly-L-lysine for 10 min after sterilization. Subconfluent cell monolayers were infected with either wild-type or mutant HSV-1 at a multiplicity of infection of 10 PFU per cell. After 1 h at 37°C, the infected medium was removed and replaced with fresh prewarmed medium until the time of harvesting. Coverslips were harvested at 2-h intervals until 16 h postinfection. Mock-infected (uninfected) cells were treated in an identical manner with the omission of virus.

RNA in situ hybridization. RNA in situ hybridization was performed by two methods.

(i) Riboprobe in situ. Cells fixed for 10 min at room temperature (RT) in 3.7% paraformaldehyde in CSK buffer {100 mM NaCl, 300 mM sucrose, 10 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)], 3 mM $MgCl_2$, 1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], pH 8} were labelled as described in the Amersham RNA Colour Kit protocol with the

omission of the proteinase K step. Fluorescein-UTP-labelled riboprobes between 200 and 400 bp in length were transcribed in vitro to produce both sense (Sp6 RNA polymerase) and antisense (T7 RNA polymerase) riboprobes. Sites of hybridization were detected with anti-fluorescein alkaline phosphatase-conjugated antibodies and alkaline phosphatase color development (Amersham kit). Digoxigenin-UTP-labelled sense and antisense riboprobes were synthesized from the same set of templates. Regions of hybridized probe were detected by using an anti-digoxigenin fluorescein-conjugated antibody at a dilution of 1:10 (Boehringer Mannheim).

A number of negative control experiments were performed. Treatment with RNase A (200 μg/ml) and RNase H (100 U/ml) at 37°C for 20 min prior to hybridization removed all signal. RNase H treatment after hybridization removed any DNA-RNA hybrids and reduced the background label but did not affect the genuine signal; RNase A treatment after hybridization removed all unhybridized probe. Treatment with DNase (100 U/ml) for 1 h prior to hybridization removed the background label but had no effects on the signal obtained.

(ii) Biotinylated RNA oligonucleotides. Cells grown as monolayers on coverslips were permeabilized with 0.5% Triton in CSK buffer for 30 s on ice prior to fixation for 10 min at RT with 3.7% paraformaldehyde in CSK buffer. Biotinylated 2′-*O*-methyl RNA oligonucleotides were hybridized for 1 h at RT at a final probe concentration of 1 pmol/μl (7) in a humidified chamber as follows. Fixed cells were rinsed in 6× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]) and incubated for 5 to 15 min with tRNA (0.5 μg/μl in 6× SSPE–5× Denhardt's solution), the probe was added in an equal volume of 6× SSPE–5× Denhardt's solution, and hybridization was for 1 h at RT. Cells were washed in 6× SSPE, and the probe was detected with Avidin-fluorescein isothiocyanate (FITC) diluted 1:500 in Avidin wash buffer (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.9], 150 mM KCl, 0.05% Tween). Coverslips were either mounted with Mowiol or double labelled by using a monoclonal anti-B″ U2 snRNP antibody and a rhodamine-conjugated secondary antibody as described in "Immunofluorescence and antibodies" below.

In situ riboprobes. Fluorescein-UTP- or digoxigenin-UTP-labelled sense and antisense riboprobes were synthesized as described in the Amersham RNA Colour Kit. Essentially, riboprobes of between 200 and 400 bp in length were transcribed in vitro by using Sp6 or T7 RNA polymerase with linearized plasmids encoding the various gene-specific probe regions as the template. Probes against the following HSV-1 gene transcripts were generated: IE63 transcriptional transactivator (genomic map positions [47] 115035 to 115435), 400-bp probe; IE110 transcriptional transactivator, exon 3 (120480 to 121033), 210-bp probe; UL15 DNA packaging protein, exon 1 (29419 to 29823), 404-bp probe; UL44 glycoprotein C (97444 to 97844), 400-bp probe; and UL38 capsid protein (86016 to 85616), 400-bp probe.

Biotinylated 2′-*O*-methyl RNA oligonucleotides kindly provided by Cruachem, Ltd., Glasgow, Scotland, were generated against U1 snRNP (biotin-CC UGC CAG GUA AGU AU) (16-mer) (7), IE110 exon 3 (biotin-UGU UAC UGC UGC CGU GU) (17-mer), and IE110 intron 1 (biotin-UAU GUG UUG GGG GUC UGU A) (19-mer). These probes were used in conjunction with immunofluorescence assays.

DNA in situ hybridization. A biotinylated poly(dT) DNA oligonucleotide (18-mer) was used to locate polyadenylated RNAs in the nucleus, using the method described for the RNA oligonucleotides.

Immunofluorescence and antibodies. Indirect-immunofluorescence experiments were performed as previously described (56). All antibodies were diluted in phosphate-buffered saline before use. The U2 splicing snRNPs were labelled with the B″ monoclonal antibody 4G3 (23) at a dilution of 1:5.

Microscopy. Labelled cells were examined with a Nikon light and fluorescence microscope. Photographs of alkaline phosphatase-labelled cells were taken with 160T Fuji films. Fluorescently labelled cells were photographed with ASA 400 Ektachrome films.

RESULTS

Intron-containing transcripts are retained in the nuclei of infected cells. HeLa cells infected with wild-type HSV-1 for 0, 2, 4, 8, and 16 h were labelled with antisense fluorescein-labelled riboprobes directed against the coding portion of IE63, an intronless transcript (Fig. 1a to d), and exon 3 of IE110, a transcript which contains two introns (Fig. 1e to h), and the hybridized probes were detected by alkaline phosphatase color development. The viral probes hybridized specifically to virus transcripts and did not label the uninfected cells (Fig. 1a and e). At 2 h postinfection, cells labelled with the anti-IE63 riboprobe demonstrated predominantly cytoplasmic label with some faint nuclear stain (Fig. 1b). As infection proceeded, labelling became more intense, with the pattern remaining constant and with IE63 transcripts being predominantly cytoplasmic at the 8-h (Fig. 1c) and 16-h (Fig. 1d) time points. The IE110 exonic riboprobe demonstrated a strikingly different labelling pattern. At 2 h postinfection, the label was largely cytoplasmic with some quantity of nuclear stain (Fig. 1f). By 4 to 6 h a large proportion of the label was found in the nucleus in distinct densely staining clumps, and cytoplasmic label was noticeably reduced (Fig. 1g). This became more pronounced, and by 8 h (not shown) and 16 h (Fig. 1h) the nucleus was packed with accumulated IE110 transcripts (somewhat obscuring the discrete clump formation), and little cytoplasmic label was seen. RNase A (Fig. 1k) and RNase H (Fig. 1l) treatments of cells labelled with an anti-IE63 riboprobe 8 h postinfection demonstrated that the hybridization was specific: RNase A removed any unhybridized probe and reduced the background label slightly; RNase H digested any RNA-DNA hybrids and removed some background label, but the bulk of the signal was unaffected. Sense riboprobes against IE110 (Fig. 1i) and IE63 (Fig. 1j) did not hybridize.

To investigate whether these two distinctive labelling patterns were exhibited by other spliced and unspliced viral RNAs, the locations of three additional viral transcripts were examined throughout infection. HeLa cells were labelled with antisense riboprobes against the late genes UL38 (Fig. 2a to c), UL44 (Fig. 2d to f), and UL15 (Fig. 2g to j). The intronless UL38 and UL44 transcripts were detected almost exclusively in the cytoplasm throughout infection (Fig. 2a to c and d to f, respectively), with very little nuclear label observed. Occasional nonspecific label was found associated with nucleoli (Fig. 2f); this could be removed by posthybridization RNase A treatment (not shown). An antisense riboprobe against the UL15 transcript, which contains a single intron, labelled infected nuclei with an intermediate nuclear and cytoplasmic phenotype (Fig. 2g to j). Throughout infection (0 to 16 h), UL15 transcripts could be detected in the nucleus in large clumps, but there was also a significant amount of cytoplasmic label, a phenotype intermediate between those of IE63 and IE110 transcripts. Sense riboprobes against UL38, UL44, and UL15 (not shown) exhibited no hybridization under these conditions. RNase A, RNase H, and DNase I treatments of the cells hybridized with the antisense UL15 probe after labelling at 8 h postinfection did not alter the labelling patterns (not shown), except that nonspecific nucleolar labelling was removed.

IE110 transcripts which lack introns are still retained in the nucleus. HeLa cells infected with the virus 110C1, in which both introns of IE110 have been precisely removed, were labelled with an antisense IE110 riboprobe from exon 3 over a time course of infection, and sites of hybridization were detected by alkaline phosphatase color development.

The mock-infected cells were not labelled with this probe (Fig. 3a). Cells labelled 2, 4, 8, and 16 h postinfection with 110C1 (Fig. 3b to e) demonstrated an intermediate phenotype, with considerably more label retained in the nucleus than observed for a transcript without introns (compare Fig. 3c with Fig. 1c) and also with more cytoplasmic label than seen with the IE110 intron-containing transcripts (compare Fig. 3c with Fig. 1g). The IE110 sense riboprobe did not label the cells (Fig. 3f), and RNase A and RNase H treatments posthybridization had no effect on the signal obtained (Fig. 3g and h).

IE63 protein is required for the nuclear retention of intron-containing viral transcripts. HeLa cells infected with the IE63 mutant virus 27-lacZ for 0, 4, 8, and 16 h (not shown) were labelled with an antisense fluorescein-labelled riboprobe directed against the coding portion of IE63 (Fig. 4a to c), IE110 exon 3 (Fig. 4d to f), and UL15 exon 2 (Fig. 4g to i), and the hybridized label was detected by alkaline phosphatase color development. Mock-infected cells were not labelled with any of the probes used (Fig. 4a, d, and g). The antisense IE63 ribo-

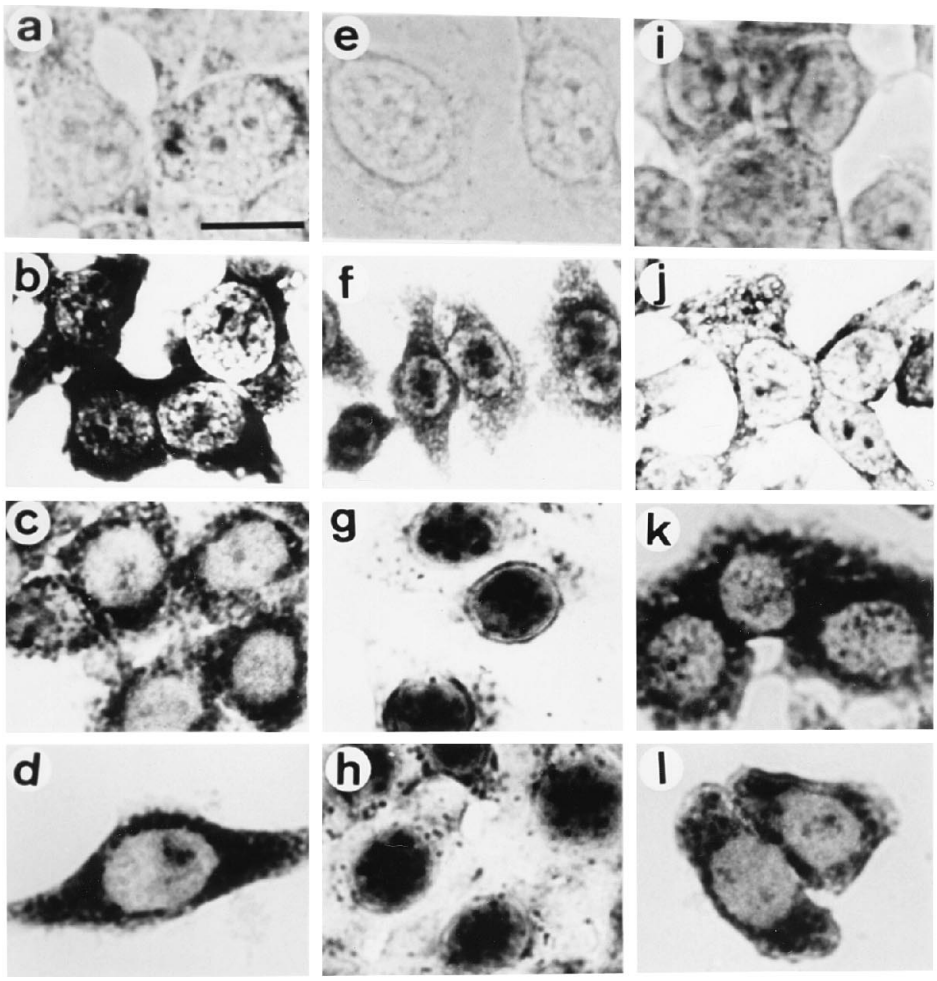

FIG. 1. Intron-containing transcripts are retained in the nucleus. Uninfected HeLa cells (a) and HeLa cells infected with wild-type HSV-1 for 2 h (b), 8 h (c), and 16 h (d) were labelled with an antisense riboprobe against IE63, and the hybridized probe was visualized by immunolabelling and alkaline phosphatase color development. HeLa cells were also labelled with an antisense riboprobe against IE110 in mock-infected cells (e) and in cells 2 h (f), 6 h (g), and 16 h (h) postinfection. As controls, cells at 16 h postinfection were labelled with sense probes against IE63 (i) and IE110 (j). Posthybridization RNase A (k) and RNase H (l) treatments of cells labelled with an antisense IE110 riboprobe 8 h postinfection were performed. Bar, 10 μm.

probe detected IE63 transcripts in the cytoplasm throughout infection (Fig. 4b and c); the IE63 sense probe did not hybridize to cells (not shown).

The IE110 antisense riboprobe labelled transcripts which were present exclusively in the cytoplasm throughout the course of 27-lacZ infection (Fig. 4e and f). The IE110 sense probe did not hybridize under these conditions (not shown). The antisense UL15 riboprobe detected all UL15 transcripts exclusively in the cytoplasm at both 4 h (Fig. 4h) and 8 h (Fig. 4i) postinfection. The UL15 sense probe did not hybridize with the cells under these conditions (not shown).

27-lacZ infection of the complementing 2-2 cell line demonstrated wild-type labelling patterns such that cells at 8 h postinfection demonstrated the distinctive nuclear retention of IE110 transcripts in large clumps (Fig. 4j). RNase A and RNase H treatments of 27-lacZ-infected HeLa cells labelled with the anti-IE110 riboprobe had no effect on the signal obtained (not shown).

The intron-containing transcripts retained in the nucleus colocalize with the redistributed snRNPs at later times postinfection. HeLa cells at 0, 8, and 16 h postinfection with HSV-1 were fixed and labelled with biotinylated RNA oligonucleotides against either the intronic or exonic regions of IE110, which was followed by Avidin-FITC detection, and with an antibody against the B″ component of the U2 snRNP, which was followed by antimouse rhodamine detection. In mock-infected cells the U2 snRNP demonstrated a normal diffuse, speckled pattern (Fig. 5b), and the IE110 exon and intron probes only weakly labelled these cells (Fig. 5a). At 8 h (Fig. 5c) and 16 h (not shown) postinfection, IE110 exonic portions of the transcript were detected in clumps within the nucleus, and redistributed snRNP clumps (Fig. 5d) predominantly colocalized with these concentrated spots of IE110 probe. Cells at 16 h postinfection double labelled with probes specific to the exonic (Fig. 5e) and the intronic (Fig. 5f) portions of the IE110 transcript demonstrated that a large proportion of the two probes colocalize.

At late times postinfection, polyadenylated RNAs are distributed in distinct nuclear spots. HeLa cells at 0, 4, 8, and 16 h postinfection with wild-type HSV-1 were labelled with a biotinylated poly(dT) DNA oligonucleotide, which was followed by Avidin-FITC detection, and with an antibody against the B″ component of the U2 snRNP, which was followed by antimouse rhodamine detection. In mock-infected cells, both

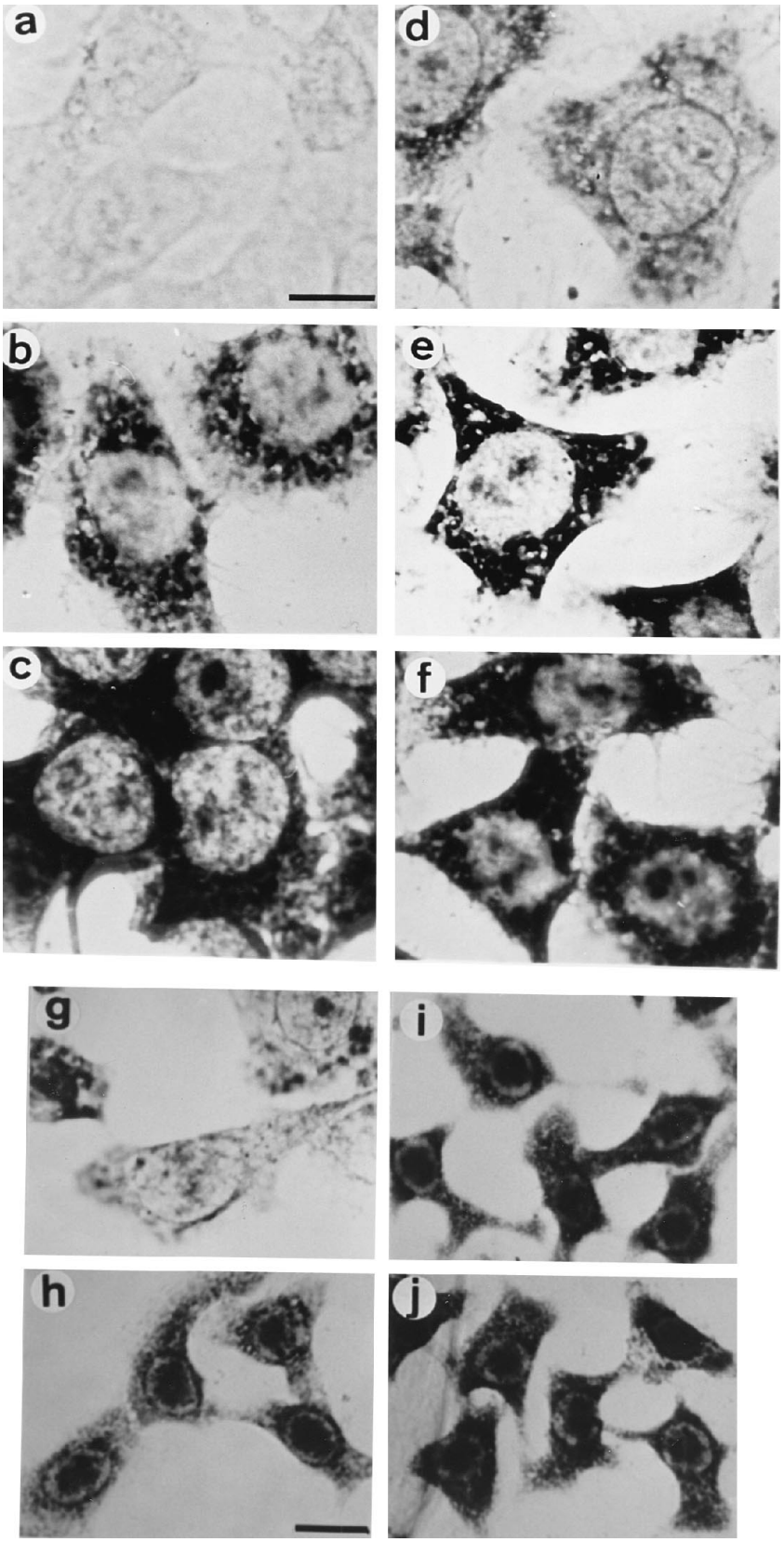

FIG. 2. Intronless transcripts are rapidly exported to the cytoplasm. Mock-infected HeLa cells (a and d) and HeLa cells infected with HSV-1 (b, c, e, and f) were labelled with antisense riboprobes against UL38 (a to c) and UL44 (d to f) at 8 h (b and e) and 16 h (c and f) post-infection. Mock-infected cells (g) and infected cells (h to j) were labelled with an antisense riboprobe against UL15 at 4 h (h), 8 h (i), and 16 h (j) postinfection. The hybridized probe was detected by alkaline phosphatase color development. Bars, 10 μm.

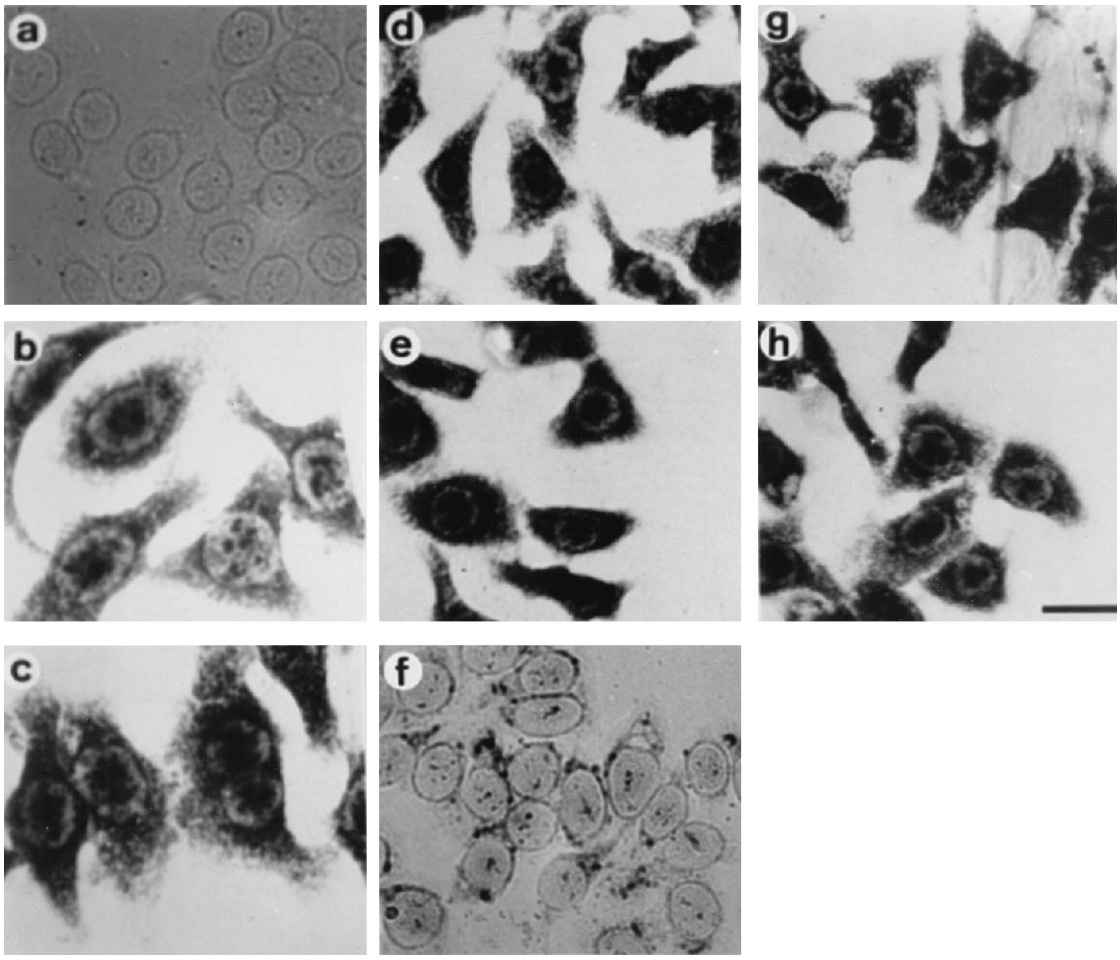

FIG. 3. Exon sequences can define the nuclear retention of IE110 transcripts. Mock-infected HeLa cells (a) and cells infected with the IE110 mutant virus 110C1 for 2 h (b), 4 h (c), 8 h (d), and 16 h (e) were labelled with an antisense riboprobe against the IE110 transcript, and the hybridized label was detected by alkaline phosphatase color development. Cells at 16 h postinfection with 110C1 were also labelled with a sense IE110 probe (f). Posthybridization RNase A (g) and RNase H (h) treatments were performed on cells 16 h postinfection, labelled with the antisense IE110 riboprobe. Bar, 10 μm.

demonstrated a diffuse, speckled labelling pattern (Fig. 6a and b) with a ringing of the dT label around the nucleoli (Fig. 6a). At 4 h the snRNPs had become clumped in appearance, but the poly(dT) label remained unaltered (not shown) except for a noticeable increase in the amount of cytoplasmic polyadenylated RNA. By 8 h postinfection, the snRNPs were quite punctate in distribution (Fig. 6d) and the poly(A)$^+$ RNA was distributed throughout the nucleus and cytoplasm (Fig. 6c). At 16 h postinfection, the snRNPs were highly punctate in distribution (Fig. 6f) and the poly(A)-containing RNA was present in a large number of brightly labelled nuclear foci in most cells and diffusely throughout the cytoplasm, but the intensity of the cytoplasm relative to the nucleus means that the cytoplasmic label is not clearly visible at the exposure shown (Fig. 6e). Only a small proportion of the poly(dT) foci colocalize with the redistributed snRNP spots. DNase treatment prior to hybridization did not remove the signal, demonstrating that the probe was RNA specific (not shown).

DISCUSSION

Cellular locations of spliced and unspliced viral transcripts. The data of Hardy and Sandri-Goldin (26) and Hardwicke and Sandri-Goldin (24) showed that HSV infection causes an inhibition of splicing both in vitro and in vivo such that pre-mRNAs accumulate in the nucleus and, further, that IE63 protein is required for this effect. Here, we have directly examined the accumulated intracellular localizations of viral transcripts which contain or lack introns and which are expressed at different times postinfection, and we have determined the involvement of IE63 in their localization. Intronless transcripts such as those from the IE63, UL38, and UL44 genes were almost exclusively cytoplasmic in location, and very little RNA was found in the nucleus, indicative of rapid nucleocytoplasmic transport. The IE110 transcript which contains two introns was detected predominantly in the cytoplasm at IE times; however, as infection proceeded from 4 to 6 h onwards, very little cytoplasmic label was detected and the nucleus became filled with accumulated transcripts present in large clumps. Double-label experiments demonstrated that intronic and exonic IE110 probes colocalized in the nucleus at later times, but whether this represents unspliced transcripts or spliced RNAs with excised introns remaining close by cannot be distinguished. Xing et al. (79) have shown that excised neurotensin introns were free to diffuse, whereas unprocessed RNAs remained associated with the nuclear substructure; thus, colocalization of the intronic and exonic IE110 probes most likely represents unspliced pre-mRNAs. The intron-containing

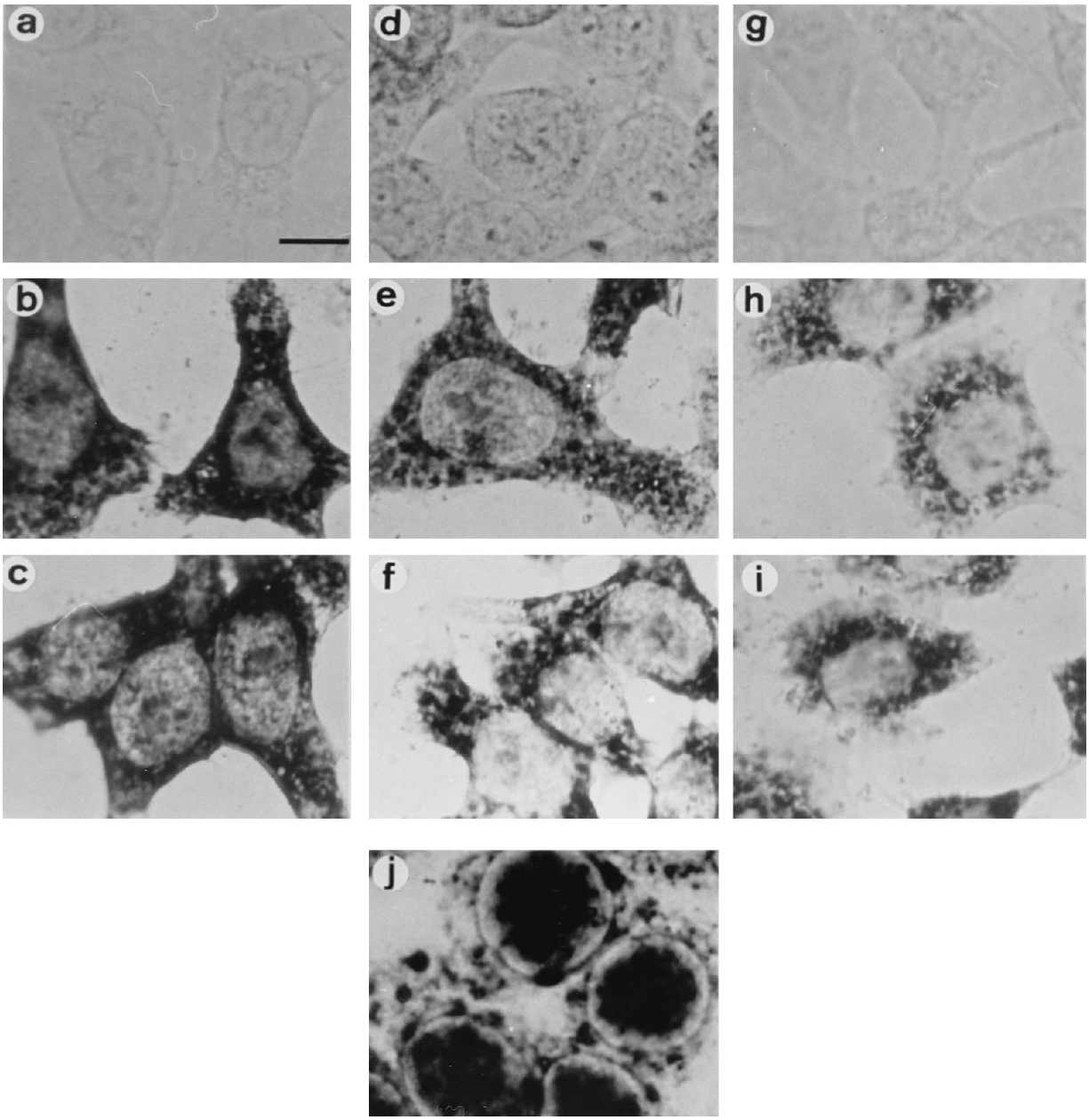

FIG. 4. IE63 is required for the nuclear retention of intron-containing transcripts. Mock-infected HeLa cells (a, d, and g) and cells infected with the IE63 mutant virus 27-lacZ for 4 h (b, e, and h) and 8 h (c, f, and i) were labelled with antisense riboprobes against IE63 (a to c), IE110 (d to f), or UL15 (g to i), and the hybridized label was detected by alkaline phosphatase color development. (j) 2-2 cells, the complementing cell line for the 27-lacZ virus, were infected with 27-lacZ for 8 h and labelled with an antisense riboprobe against IE110, and the label was detected by alkaline phosphatase color development. Bar, 10 μm.

transcript from the UL15 late gene cannot be detected at IE times, but once detected it was present in both the nucleus and the cytoplasm throughout infection, forming the characteristic nuclear clumps. Infections with virus 27-lacZ, during which splicing is not inhibited, unambiguously demonstrated that IE63 was required for the nuclear retention of intron-containing transcripts, since in the absence of IE63, all transcripts were rapidly transported to the cytoplasm.

Intron-containing transcripts retained within the nucleus colocalize with the redistributed snRNPs. HSV-1 infection (44), and more specifically IE63 expression (56), caused a redistribution of the splicing snRNPs away from their diffuse, speckled nuclear pattern to a highly punctate organization, and IE63 colocalized with these snRNP clusters. Sandri-Goldin et al. (67) have shown that the C terminus of IE63 is required for this snRNP redistribution effect, implicating this as a candidate region for causing the nuclear retention of intron-containing transcripts.

Intron-containing IE110 transcripts retained within the nucleus colocalized with the redistributed snRNPs. A possible explanation is that the punctate snRNPs are active in splicing and intron-containing transcripts are recruited there for processing prior to nuclear export; thus, the sites of transcript accumulation represent processed but nontransported RNAs. Alternatively, intron-containing transcripts may localize to the snRNP clumps through an affinity of splicing snRNPs for

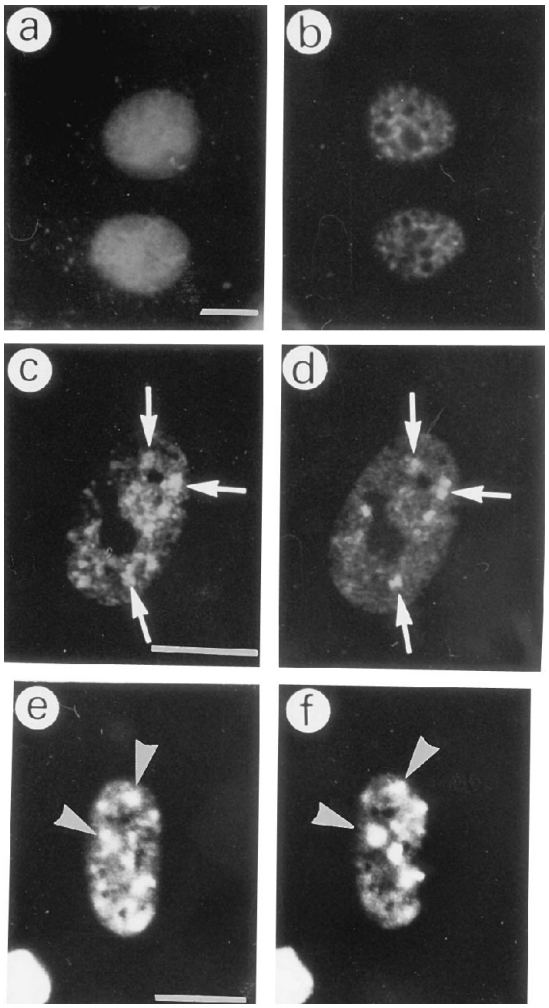

FIG. 5. The intron-containing transcripts retained in the nucleus colocalize with the redistributed snRNPs. Mock-infected HeLa cells (a and b) and cells at 8 h postinfection with HSV-1 (c and d) were double labelled with a biotinylated 2′-*O*-methyl RNA oligonucleotide against IE110 exon 3 (a and c) and an antibody against the B″ component of the U2 snRNP (b and d); the probe and antibody were detected with FITC and Texas Red, respectively. HeLa cells at 16 h postinfection with HSV-1 were double labelled with a biotinylated IE110 exonic RNA oligonucleotide (e) together with a digoxigenin-labelled IE110 intronic probe (f); the hybridized probes were detected with Texas Red and FITC, respectively. Arrows and arrowheads indicate areas of colocalization. Bars, 10 μm.

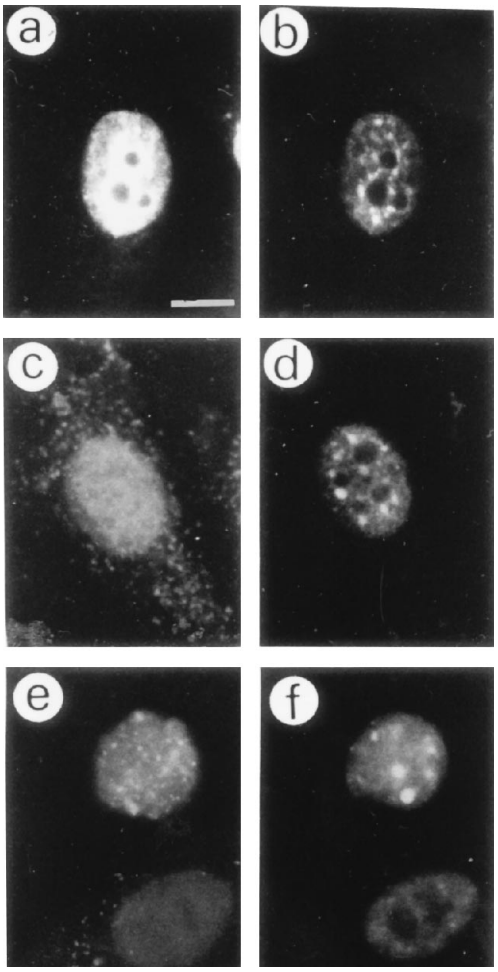

FIG. 6. Polyadenylated RNAs accumulate in the nucleus during the course of infection. Mock-infected HeLa cells (a and b) and cells at 8 h (c and d) and 16 h (e and f) postinfection with HSV-1 were double labelled with a biotinylated poly(dT) oligonucleotide (a, c, and e) and an antibody against the B″ component of the U2 snRNP (b, d, and f); the two labels were detected with FITC and Texas Red, respectively. Bar, 10 μm.

RNA, but since the snRNPs are now clumped into nonfunctional storage sites, trapped RNAs which cannot be spliced or transported accumulate. Using an anti-interchromatin granule antibody, Besse et al. (1) have shown that the redistributed snRNPs are condensed into these regions, which are believed to function only as storage sites, not as active sites of processing or transcription. We favor the view that the redistributed snRNPs are inactive in splicing, and we have shown by direct labelling methods that these clumps are not coincident with sites of viral transcription or viral DNA replication (unpublished results). Mermoud et al. (52) and others have identified changes in the phosphorylation state of splicing factor SR domains which target RNA-processing proteins, and this may trigger the movement of splicing factors between storage and active sites (22, 74). Sandri-Goldin et al. (67) have data which suggest that while the IE63-mediated impairment of host cell splicing correlates with the redistribution of the snRNPs, these alterations are not sufficient to fully inhibit splicing in vitro or splicing of introduced reporter constructs. Their test conditions detected splicing in the presence of redistributed snRNPs, suggesting that either the redistributed snRNPs were active or sufficient snRNP remained diffusely distributed in the nucleoplasm to perform the amount of splicing required. Malim and Cullen (43) suggest that the cellular factors most probably responsible for the nuclear retention of pre-mRNAs are splicing factors, and it has been found that IE63 affects the phosphorylation states of at least two cellular proteins, one of which appears to be the 70-kDa component of the U1 snRNP (66). This could represent the mechanism whereby IE63 promotes the snRNP redistribution effect.

The fate of spliced transcripts. The UL15 late transcript, which contains a single intron, is present in both the nucleus and the cytoplasm throughout infection, forming the characteristic nuclear clumps. Since virus-induced inhibition of splicing is well under way by the time UL15 transcripts are made, these transcripts are immediately susceptible to the nuclear retention effect; however, at later times a considerable propor-

tion of UL15 transcripts is found in the cytoplasm, in contrast to the situation found with the IE110 transcripts. Hardy and Sandri-Goldin (26) showed that 30% of cytoplasmic UL15 RNA at late times was unspliced, suggesting that there are less-stringent controls governing the export of UL15 than governing that of IE110. Everett et al. (15) have identified a truncated form of the IE110 protein during wild-type infection, which arises from a failure to remove the second intron, with the result that an in-frame stop codon within this intron terminates translation. This suggests the possibility of alternative or aberrant splicing events affecting IE110 expression. It is possible that the differences in localization of IE110 and UL15 transcripts relate directly to the time postinfection of their expression; however, while IE transcription peaks at around 3 to 4 h postinfection, there is a low level of IE transcription and a persistence of transcripts throughout infection (27, 78), with the result that some IE110 transcription would be occurring at the time of UL15 expression. Another consideration is the fact that IE110 contains two introns as opposed to one for UL15; this could result in IE110 transcripts being more tightly retained in the nucleus, but such a theory has not yet been proven.

Exon sequences cause the nuclear retention of intron-containing transcripts. The distribution of IE110 transcripts in cells infected with the mutant virus 110C1, in which both introns of IE110 have been precisely removed, indicates that exonic sequences are dictating the interaction of this transcript with the splicing snRNPs. A considerable amount of IE110 transcripts was detected in the cytoplasm throughout infection with 110C1; however, the IE110 exonic probe labelled the nuclei in large spots from early times, a pattern different from that shown by the wild-type IE110 intron-containing transcript, in which very little cytoplasmic RNA was present even at early times. Niwa et al. (53) suggest that the exon rather than the intron is the unit of recognition in vertebrate splicing; factors binding 3′ and 5′ splice sites communicate across exons to define them as the first units of spliceosome assembly, which precedes the definition of introns for removal. Sequences located within an exon downstream of a 3′ splice site can facilitate initial spliceosome complex formation, and a number of such preassembly complexes have been identified (63). Liu and Mertz (40) have demonstrated that selection of, but not necessarily cleavage at, a polyadenylation site precedes excision of the 3′-terminal intron in vivo, further supporting the exon definition theory. snRNP recognition of IE110 transcripts from the virus 110C1 must be due to exonic sequences.

Polyadenylated RNAs accumulate in the infected-cell nucleus. To examine the localization of viral and cellular polyadenylated RNAs, we utilized a poly(dT) in situ hybridization procedure. In uninfected cells and up to 6 to 8 h postinfection, the poly(A)-containing RNA was distributed widely throughout the nucleoplasm and the cytoplasm. At later times, a large number of spots of accumulated poly(dT) were seen in the nucleus, and only a small proportion of these colocalized with the redistributed snRNPs. These colocalized sites represent polyadenylated (possibly intron-containing) transcripts which are interacting with snRNPs; the remaining poly(A)$^+$ RNA spots represent RNAs awaiting transport or a stable poly(A)$^+$ population retained in the nucleus. A similar reorganization of poly(A)$^+$ RNA was observed by Huang et al. (31) when RNA polymerase II activity was inhibited and by Visa et al. (76) following heat shock treatment of cells; Huang et al. (31) proposed that the nuclear population of polyadenylated RNAs may serve some unknown functional role.

Other viral proteins with comparable posttranscriptional effects. IE63 is a complex protein with regulatory functions affecting virus transcription, RNA processing, and DNA replication. Its posttranscriptional functions have counterparts in other viral systems. During influenza virus infection, the snRNP and associated factors become more punctate in distribution, and Fortes et al. (20) determined that the NS1 protein was essential for this effect. NS1 causes an inhibition of splicing of its own mRNA (42, 60) with a resulting accumulation of splicing intermediates (19) and has been found to induce a generalized block of mRNA nucleocytoplasmic transport (19, 59). Like that of HSV-1, the influenza virus genome contains few introns (20); hence, an inhibition of splicing primarily affects the host. In contrast, the HIV type 1 Rev protein promotes the export of partially spliced or nonspliced transcripts to the cytoplasm in a sequence-specific manner by binding directly to the Rev response element present in intron-containing RNAs, in conjunction with a cellular cofactor, Rab (3). Recently, Diaz et al. (12) have shown that the Us11 late HSV-1 protein can functionally substitute for HTLV Rex, and to a lesser extent for HIV type 1 Rev, in promoting the cytoplasmic accumulation of incompletely spliced HIV RNAs while cellular mRNA remains in the nucleus. In HSV-infected cells, Us11 is packaged into virions, and its precise function is unclear; whether Us11 can promote the transport of HSV-1 transcripts remains to be determined but could account for the cytoplasmic accumulation of unspliced UL15 transcripts also expressed at late times.

In conclusion, the HSV-1 IE63 protein causes the nuclear retention of or a failure to export intron-containing IE110 and UL15 transcripts throughout infection; intronless transcripts are unaffected by the presence or absence of IE63. Furthermore, these nuclear accumulated transcripts colocalize with the redistributed cellular splicing snRNPs but represent only a small proportion of total nuclear polyadenylated RNAs. Elucidation of the mechanisms by which the IE63 protein carries out so many functions and of its involvement in the nucleocytoplasmic transport of viral transcripts provides an interesting challenge for future work.

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