Redistribution of Nuclear Ribonucleoprotein Antigens during Herpes Simplex Virus Infection

Terence E. Martin, Stephen C. Barghusen, George P. Leser, and Patricia G. Spear
Department of Molecular Genetics and Cell Biology, The University of Chicago, Chicago, Illinois 60637

Abstract. Infection of human epidermoid carcinoma No. 2 cells with herpes simplex virus type 1 (HSV-1) leads to a reorganization of antigens associated with both the small and heterogeneous nuclear ribonucleoprotein complexes (snRNP and hnRNP). The hnRNP core protein antigens remain associated with the host chromatin, which appears to collapse into internal aggregates and along the nuclear envelope. More striking is the formation of prominent clusters of snRNP antigens (both general and U1 snRNP specific), which appear to condense throughout the nucleus then migrate to the periphery. These snRNP clusters have been identified at the fine structure level by immuno-electron microscopy. The HSV-1 presumed transcriptional activator ICP4, DNA-binding protein ICP8, and two capsid proteins ICP5 and p40 are not detectably associated with the snRNP clusters. Similar reorganization of snRNP occurs with HSV-2 and upon infection of African green monkey VERO cells with HSV-1. We speculate that the snRNP clusters arise from an increase in size and density of the interchromatin granule region of the host cell as a result of the partial inactivation of snRNP and host pre-mRNA splicing.

The recent availability of specific antibodies and refined immunocytochemical techniques has provided the opportunity for a more detailed study of the cell and molecular biology of macromolecules associated with gene expression in eukaryotic cells. Of particular interest are those associated with the transcription and processing of mRNA molecules in the cell nucleus. These include the proteins that bind to pre-mRNA in hnRNP complexes (reviewed by Martin et al., 1980 and Dreyfuss, 1986) and the RNA and protein components of snRNP, which are now firmly implicated in the processing of mRNA precursors, including the splicing and 3' maturation steps (see Sharp, 1987 for a recent review).

Using immunocytochemical procedures at both light and electron microscope levels, we have previously been able to demonstrate that the core proteins of hnRNP complexes and a fraction of the snRNP antigens co-localize at intranuclear sites which are associated with RNA synthesis (Martin and Okamura, 1981; Fakan et al., 1984). More recently, we have detected both hnRNP proteins, as expected, and snRNP antigens on nascent RNA polymerase II transcripts observed in Miller spreads of eukaryotic chromatin (Fakan et al., 1986). Thus, there is a direct association of these readily detectable nuclear components with the normal transcription-processing machinery of the eukaryotic cell. We have therefore sought biologically significant factors which may have major effects on these essential nuclear complexes. One of the more extreme morphological changes that we have observed is induced in primate cells by infection with herpes simplex virus (HSV)1, and we have attempted to characterize some aspects of the phenomenon in this paper.

Pertinent aspects of the replication cycle of this herpes virus have been summarized recently (Roizman and Batterson, 1986). Briefly, entry of the virus into a cell results in transport of the DNA genome to the cell nucleus. Here the genome is transcribed by cell RNA polymerase II under the influence of viral regulatory factors, some of which are carried in by the input virus and some produced de novo after infection. Except that most HSV mRNAs do not require splicing, nothing unusual about processing or transport of viral transcripts has yet been described. Most viral proteins are transported to the nucleus where they participate in viral DNA replication, regulation of transcription, and packaging of progeny genomes into icosahedral nucleocapsids. Viral glycoproteins become incorporated into the membranes of infected cells, including the nuclear membrane. Nucleocapsids acquire their envelopes by budding through the inner nuclear membrane. Progeny virions are then transported out of the cell via the Golgi apparatus.

The transcription of viral genes is regulated (reviewed by Roizman and Batterson, 1986). In the absence of de novo viral protein synthesis, only the immediate early (α genes) can be transcribed. One or more of the α proteins then enables transcription of the early (β) genes to occur. Similarly, α or

1. Abbreviations used in this paper: BrdU, bromodeoxyuridine; chrp, core hnRNP proteins; HEp-2, human epidermoid carcinoma No. 2; HSV-1, herpes simplex virus type 1; PLP, periodate-lysine-paraformaldehyde.
Materials and Methods

The Journal of Cell Biology, Volume 105, 1987 2070

tinued incubation until the time for fixation. The virus strains used were

key kidney (Vero) cells were grown on glass coverslips in DME with 10%

at room temperature.

paraformaldehyde (PLP) fixative of McLean and Nakane (1974) for 15 rain

Fixation

were used at a dilution of 1:2 and undiluted, respectively. Purified monoclo-

Antibodies Y12, which reacts predominantly with a 28-kD polypeptide

HSV-I(HFEM), HSV-I(KOS), and HSV-2(G).

Cells and Virus

is greatly altered by HSV infection is therefore of consider-

cation on production of particular cell mRNAs are now under

rRNA precursors to a larger extent than transcription of the

cription, processing, and transport have not been indepen-
dantly assessed in most cases. Early studies on preribosomal

showed that HSV infection inhibited processing of

RNA to a larger extent than transcription of the

dNA (Wagner and Roizman, 1969). Effects of HSV infec-

on production of particular cell mRNAs are now under

vestigation, with few results available as yet. It is possible

that as for pre-rRNA the correct processing of host pre-
mRNA may become defective in HSV-infected cells. Our

finding that the nuclear distribution of host snRNPs antigens is
greatly altered by HSV infection is therefore of consider-
able potential significance.

Materials and Methods

Cells and Virus

Human epidermoid carcinoma No. 2 (HEp-2) cells or African green mon-
key kidney (Vero) cells were grown on glass coverslips in DME with 10%

FBS, or medium 199 with 5% FBS, respectively. The cells were inoculated

with virus at 10⁶ plaque-forming units per ml or 10 plaque-forming units

per cell input in medium 199 with 1% FBS. The virus added was sufficient

to infect >80% of cells in all experiments. After incubation for 2 h, the inoc-

ula were removed and fresh medium 199 with 1% serum added for con-
tinued incubation until the time for fixation. The virus strains used were

HSV-1(HFEM), HSV-1(KOS), and HSV-2(G).

Monoclonal Antibodies

Anti-HSV protein antibodies, 39S (anti-DNA binding protein ICp8), 58S

(anti-immediate-early protein ICp4), 74S (anti-capid protein p40), and

102S (anti-capid protein ICp5), were supplied as ascitic fluid by M. Zweig

(Showalter et al., 1981) and were used at a dilution of 1:50. Antibody ID2,
specific for the hnRNP core polypeptides (Leser et al., 1994), was an am-

monium sulfate fractionation of ascitic fluid and used at a dilution of 1:100.

Antibodies Y12, which reacts predominantly with a 28-kD polypeptide

found in the U₁, U₂, U₃, U₄, and U₅ snRNPs (Lerner et al., 1981), and

RNP, specific for the 70-kd polypeptide RNP antigen associated with the

U₁ snRNP complex (Billings et al., 1982), were culture supernatants and

were used at a dilution of 1:2 and undiluted, respectively. Purified monoclo-
nal antibodies specific for bromodeoxyuridine (BrdU) were purchased from

Becton Dickinson & Co., (Mountain View, CA) and used at a 1:25 dilution.

Fixation

The cells were washed briefly with PBS and then fixed with either 3.6%

formaldehyde in 90% acetone for 15 min on ice, or the peridate-lysine-

paraformaldehyde (PLP) fixative of McLean and Nakane (1974) for 15 min

at room temperature.

Indirect Immunofluorescence

The cells were reacted with the primary antibody for 30 min at room

temperature, washed in PBS for 30 min, and then reacted with a fluorescein-

labeled secondary antibody also for 30 min at room temperature. All

bodies, with the exception of RNP, which was used undiluted, were diluted

to an appropriate concentration with a buffer of 1% BSA, 0.5% Triton X-100,

and 0.5% Na deoxycholate in PBS. After staining, the cells were washed in

PBS for 30 min and mounted on slides using a 67% glycerol/33% PBS

mounting medium with 5% n-propyl gallate added to reduce fading.

Determination of DNA Distribution by DAPI Stain

After antibody reaction, cells were stained with the DNA stain DAPI (Wil-

liamson and Fennell, 1975), except in the case of the anti-BrdU antibody

where DAPI staining was performed before antibody reaction for reasons

discussed below. DAPI was diluted 1:5,000 in distilled water, and applied

for 30 min at room temperature. Coverslips were mounted using the above

mounting medium but omitting the n-propyl gallate, which fluorescent under

the UV filter used for DAPI visualization.

Determination of Sites of DNA Synthesis by BrdU Incorporation

Cells were labeled with BrdU (10 μM final concentration) for 20 min im-

ediately before fixation. The cells were fixed with 10% TCA in 90% etha-

tol for 15 min on ice. Subsequently, cells were treated with 0.07 N NaOH

for 2 min on ice to denature the DNA before reaction with the anti-BrdU

antibody. When a DAPI/BrdU double label was desired, the cells had to first

be stained with DAPI and photographed before they were treated with

NaOH, which prevents the subsequent observation of a distinct DAPI

pattern.

Conventional Electron Microscopy

HEp-2 cells were grown in petri dishes, and infected as detailed above. At

the indicated times postinfection, the cells were washed with PBS (pH 7.2)

and fixed at room temperature with freshly prepared 2% glutaraldehyde in

0.1 M phosphate buffer (pH 7.2). After 30 min the cells were washed with

0.1 M phosphate buffer (pH 7.2) and postfixed with 1% osmium tetroxide

in 0.1 M cacodylate buffer (pH 7.2). Then the cells were washed with caco-
dylate buffer and scraped off the surface of the petri dish using a rubber

plunger from a disposable syringe. Finally the cells were dehydrated

through a graded ethanol series and propylene oxide, and embedded in Epon

according to standard protocols. Thin sections were collected on parlondion-

coated copper grids and stained with uranyl acetate and lead citrate. Speci-

mens were examined in a Philips 101 electron microscope operating at 80

kV and equipped with a 30-μm objective aperture.

Immunoelectron Microscopy

HEp-2 cells grown and infected as described above were fixed with PLP

at 4°C. The washed cells were collected and dehydrated through a graded

ethanol series. The cells were then infiltrated and embedded in Lowicryl

K4M according to the manufacturer's instructions, except that 0.3% diben-

zoyl peroxide was substituted for the initiator and polymerization was car-

ried out in gelatin capsules at 60°C for 1-2 d. Thin sections were collected on

parlodion-coated nickel grids. Colloidal gold of defined size was pre-

pared using the sodium citrate/ascite acid procedure (Slott and Geuze,

1985). Rabbit anti-chicken IgG (Miles Laboratories, Inc., Naperville, IL)

or goat anti-mouse IgG + IgM (The Jackson Laboratory, Bar Harbor, ME)

antibodies were coupled to colloidal gold according to published procedures

(Keoghgen and Ackerman, 1977; and DeMay et al., 1981). Grids were

floated sections down upon a drop of 1% ovalbumin in Tris-buffered saline

(pH 7.2) for ~30 min. Then the grids were briefly drained and placed upon

drops of the primary antibody for 2 h. In the case of monoclonal antibodies

the hybridoma culture supernatant was used undiluted. The hnRNP-specific

(chp) chicken polyclonal serum was diluted 1:250 in Tris-buffered saline

containing 1% BSA. Where sections were double-labeled the grids were in-
cubated with both antibodies simultaneously. The grids were washed drop-

wise with ~5 ml Tris-buffered saline (containing 1% BSA), drained, and

placed upon a drop of the appropriate secondary antibody coupled to collo-
dal gold (the gold-coupled secondary immunoglobulins were diluted to 1:50

in Tris-buffered saline containing 1% BSA). After incubating for 1 h, the

grids were washed as before and finally rinsed drop-wise with 5 ml distilled

The Journal of Cell Biology, Volume 105, 1987 2070
Results

Reorganization of snRNP Antigens in HSV-infected Cells

Because infection of human cells by HSV alters the expression of host genes and the structure of the nucleus of the infected cell, we initiated studies of the localization of nuclear ribonucleoprotein complexes in this system. The most striking example of a reorganization of nuclear components has been obtained for the snRNP complexes, which are regarded as essential to the correct processing of host mRNA precursors. An example of the changes occurring in the localization of the Sm antigen associated with most snRNP complexes, excepting U₃ snRNP, a nucleolar component, is shown in Fig. 1. During the replication cycle of HSV-1 in HEp2 cells, the host nuclei increased in size and the normal morphology visible in the light microscope was drastically altered. There was a general decrease in phase contrast and disruption of the nucleoli; these changes were most apparent after 4 h from time of infection (Fig. 1, phase-contrast micrographs). More notable, however, was the changing pattern of the snRNP Sm antigen distribution. Indirect immunofluorescence localization of this antigen in uninfected cells (Fig. 1, M) revealed a speckled and diffuse pattern typically found for most mammalian cells (Tan, 1979). By 4 h after infection it was apparent that more prominent speckles or clusters were forming within the nuclei. During the next 12 h of infection, the snRNP clusters became increasingly prominent and migrated to the periphery of the enlarged nuclei with a general reduction in the diffuse snRNP component (Fig. 1, 8h, 16h). While there were many phase-dense granules in the infected cell nucleus, not all (even the most prominent) were stained by the anti-Sm snRNP antibody.

To define further this effect of HSV infection on snRNP, we examined the localization of a specific RNP complex, the U₃ snRNP known to be involved in the recognition of the 5' splice junction of mRNA precursors (Mount et al., 1983). This was performed using a mouse autoimmune monoclonal antibody specific for the 70-kD protein characteristic of U₃ snRNP complexes (Billings et al., 1982) Although the immunofluorescence signals obtained were lower with this antibody, essentially the same initial distribution, progressive change, and final localization were observed as with the more general Sm antibody (results not shown).

Reorganization of hnRNP Core Protein Antigens

In view of the major redistribution of snRNP antigens in HSV-infected cell nuclei it was important to study other components of the host mRNA transcription-processing system. The hnRNA, including pre-mRNA, of vertebrate cells is found in hnRNP complexes of which a large part of the protein component consists of a group of 33–40 kD polypeptides (reviewed by Martin et al., 1980, and Dreyfuss, 1986) termed A and B polypeptides for He La cells by Beyer et al. (1977). We had previously prepared both polyclonal and monoclonal antibodies to the major basic A and B core polypeptides (chrp's) of hnRNP complexes (Jones et al., 1980; Leser et al., 1984). These polypeptides associate with vertebrate RNA polymerase II transcripts in the nascent state (Martin and Okamura, 1981; Fakan et al., 1986).

For these studies we used a mouse monoclonal IgM antibody (I2D) which reacts strongly with all members of the basic core group of hnRNP proteins. Since we had previously observed that the accessibility of nuclear antigens to this IgM antibody required a greater permeabilization of the cells during fixation, we used an acetone-based fixative in the initial experiments. If anything, the decreased matrix density of the infected cell nuclei was more apparent with this fixative (Fig. 2, phase contrast), probably because of the collapse of the fine structure under these conditions. However, the localization of snRNP antigens by immunofluorescence of formaldehyde-acetone-fixed cells was identical to that in Fig. 1 (results not shown).

Localization of the hnRNP chrp antigens during HSV infection was very different from that of the snRNP, and segregation of the snRNP and hnRNP antigens increased with time (Fig. 2; cf. Fig. 1). The chrp polypeptides appeared to be primarily located on large collapsed structures with some lesser accumulation at the nuclear periphery (but in a very different pattern than that of the clusters of snRNP antigens).

While it had been our experience that the IgM anti-chrp antibody was unable to penetrate normal interphase nuclei of cells fixed by PLP method (Leser et al., 1984), we wished to use the fixative for subsequent electron microscope immunocytochemistry since it preserves fine structure. We therefore attempted immunofluorescence on HSV-infected HEp-2 cells fixed in this way (Fig. 3). As can be seen in mock-infected controls (M) only mitotic cells were stained; note that the chromosomes did not stain. However, during the course of infection increasing numbers of the fixed cell nuclei became permeable to the IgM antibody (4h, 8h) until essentially all cells were stained (16h). We interpret this result to indicate an increasing fragility of the nuclear envelope during the progress of HSV infection. Perhaps more important was the observation that even with the attempt to preserve fine structure to a greater degree, large clumps of hnRNP antigen-containing material was observed (most notably at 16 h in the examples shown in Fig. 3). The identical large nuclear structures were also stained in double label experiments by the DNA-specific DAPI stain (results not shown; examples of DAPI stain are given in Figs. 4 and 5).

Localization of HSV-specific Antigens in Infected Cells

The experiments described above have documented the location of host cell components involved in normal mRNA transcription and processing. Given the striking reorganization of these during HSV infection, it was important to determine the location of viral antigens and functions. It was reasonable to hypothesize, for example, that host hnRNP proteins and/or snRNP might be concentrated at sites of viral activity. This possibility was rendered unlikely by the localization with specific monoclonal antibodies of a variety of viral proteins representing different functions in the progress of infection (Fig. 4).

The viral α protein ICP4 is an immediate early viral gene product thought to be involved in regulation of viral tran-
Figure 1. Reorganization of the snRNP sn antigens during HSV infection. Phase-contrast and indirect immunofluorescent photomicrographs of HSV-1-infected HEp-2 cells fixed with PLP: mock (M), 4, 8, and 16 h after infection, stained with anti-snRNP antibody Y12.

The HSV 13 protein ICP8 is a delayed early viral gene expression and therefore presumably localized to sites of active viral genomes. The staining of infected cells by an ICP4-specific antibody demonstrated its early appearance, initially in confined regions and subsequently more generally throughout the infected nucleus, but not in the regions of highest DNA concentration (Fig. 4: ICP4, 4h, 8h; D, 8h). There was no obvious overlap of ICP4 with the snRNP clusters or the high density hnRNP core protein structures. This is one of the more significant results of our comparative study of antigen localization, indicating a segregation of viral transcription factors from those nuclear domains containing the highest concentration of RNP components presumed for host cell mRNA synthesis and processing.

The HSV β protein ICP8 is a delayed early viral gene
product and a major viral DNA binding protein. While ICP8 may have a transcriptional role, it is generally considered to function in viral DNA replication. Our studies with a monoclonal antibody specific for ICP8 gave essential agreement with previous studies (for example, Quinlan et al., 1984), indicating an initial restricted localization of the protein in the nucleus of the infected cell (Fig. 4, ICP8, 4h) which later formed much larger domains with a characteristic punctate pattern but did not overlap the regions of greatest DNA density (Fig. 4, ICP8, 8h; D 8h). There was no obvious relationship of the location of the viral DNA-binding protein to the distributions of host snRNP or hnRNP antigens shown above.

Two late viral gene products, p40 and ICP5, both capsid proteins, were also examined. At mid-infection (8 h) these were found to be distributed in large intranuclear domains similar in form to those of ICP4 and ICP8 (Fig. 4, p40, 8h; ICP5, 8h). In late infection, intensely stained zones were observed at the nuclear periphery not overlapping with highest DNA density (Fig. 4, 16h). While somewhat similar in general distribution to the host snRNP clusters, these viral capsid zones were different in shape and precise location, and probably represent at the immunofluorescence level the accumulations of viral particles observed at the nuclear envelope by electron microscopy (Nii et al., 1968; Schwartz and Roizman, 1969).

**Sites of DNA Synthesis in HSV-infected Cells**

Previous immunofluorescence studies had described the punctate distribution of the HSV DNA-binding protein ICP8 in infected cells (Quinlan et al., 1984). Results from the same laboratory also suggested that this could represent an intermediate state in the localization of the protein preceding DNA synthesis (Knipe and Spang, 1982). Other workers had attempted to locate the sites of viral DNA synthesis by radioautography at the light and electron microscope level in infected cells pulse-labeled with [3H]thymidine (Rixon et al.,...
To explore further the relationship with host cell RNP antigens we also determined the site of viral DNA synthesis, but in this case using an immunofluorescence technique that enabled direct comparison with the various antigens we could locate. We therefore pulse-labeled cells at various times after infection with the thymidine analogue BrdU and subsequently located the incorporated nucleoside with a specific monoclonal antibody (Gratzner, 1982).

Mock-infected cells pulse-labeled in this way showed the expected pattern, in which 20–30% of the population, that is those cells in S phase, were labeled (Fig. 5, M, BU). The label was generally in a diffuse or fine grain pattern which
Figure 4. Location of viral proteins ICP4, ICP8, p40, and ICP5. Indirect immunofluorescent photomicrographs of HSV-1-infected HEp-2 cells fixed with PLP and reacted with antibodies to HSV viral proteins: anti-HSV immediate-early ICP4 protein antibody (58S) at 4 and 8 h after infection, respectively, with DAPI (D) pattern for cells at 8 h. Anti-HSV DNA-binding protein ICP8 antibody (39S) at 4 and 8 h, respectively, with DAPI (D) pattern for cells at 8 h. Anti-HSV capsid protein p40 antibody (74S) at 8 and 16 h, respectively, with DAPI (D) pattern for cells at 16 h. Anti-HSV capsid protein ICP5 antibody (I02S) at 8 and 16 h, respectively, with DAPI pattern for cells at 16 h.
in some cases overlapped the total DNA distribution and in others did not (Fig. 5, M, BU; M, D). The apparent discrepancy may simply be a reflection of different stages of S phase in the particular cells; the more condensed chromatin is generally replicated late in S (Brown, 1966). The effect of HSV infection was to increase the fraction of the cell population labeled with BrdU. This was noticeable from 6 h after infection and increased until almost all cells were engaged in extensive DNA synthesis at 12 h. For the most part the BrdU-labeled DNA did not correspond to the highest DNA density (cf. BU and D on some cells in Fig. 5), but did show a marked punctate pattern similar to that characteristic of ICP8 distribution (cf. BU and ICP8 in Fig. 5).

While these results suggest that viral DNA synthesis takes place in rather discrete units, it occurs throughout domains within the nucleus and not at the periphery in association with bulk DNA nor the snRNP clusters described above.

Effect of Other Agents on the Distribution of snRNP in HEp2 Cells

Our studies with inhibitors on other mammalian cells indicated that the reorganization of snRNP and hnRNP antigens we observed in HSV-1-infected HEp2 cells was probably not the result of a simple general inhibition of host RNA and protein synthesis. Nevertheless we tested this possibility using a number of agents to alter host HEp2 cell function. Neither the enlarged and peripheral clusters of snRNP antigens nor the "collapsed" hnRNP-containing structures were induced by cycloheximide, actinomycin, or heat shock (results not shown). Further, the other human cell lines we have studied after adenovirus infection did not exhibit the changes characteristic of HSV-1 infection.

That similar clustering of snRNP antigens can be caused by other herpes viruses was demonstrated by the infection of HEp2 cells with HSV-2 (results not shown). After 4 h of infection, prominent clusters of the snRNP Sm antigen were observed within the infected cell nucleus. However, there did appear to be some differences in the later stages of infection with the two viruses, namely, the clusters formed in HSV-2-infected cells tended to remain within the intranuclear region rather than migrate to the periphery as with HSV-1.

Other experiments not illustrated here demonstrated that the clustering of snRNP complexes and subsequent peripheral nuclear localization is not limited to the HFEM strain of HSV-1 nor to human cells since the effect was observed with HSV-1(KOS) and African green monkey VERO cells.

Fine Structure of HSV-1-infected HEp2 Cells

Electron microscopy revealed that HSV infection caused a reorganization of the fine structure of the nucleus. In particular, at later times there was a significant reduction in the staining density of the internal interchromatin regions (Fig. 6). The densely staining host chromatin (c) was primarily restricted to a close association with the envelope of the greatly enlarged nucleus, with the exception of chromatin extending into the nuclear space associated with the residual nucleolar structure. The distribution of host chromatin at the ultrastructural level was as expected from light microscope DAPI stain (Fig. 2). In addition to the appearance of viral particles (v), dense bodies (db) were often found throughout the nuclei of herpes-infected cells. The origin of dense bodies after infection is not clear, although they have been reported to contain at least one species of nucleolar protein (Puvion-Dutilleul et al., 1985). Electron-dense particles were sometimes found surrounding the dense bodies (Fig. 6, x). The nature of these particles is not known, although Puvion-Dutilleul et al. have suggested on the basis of their morphology that they are cores of viral nucleocapsids.

The nuclei of HSV-infected cells also contained a number of large clusters of ~15-nm granules. These granule clusters (gc) frequently appeared to be associated with the periphery of chromatin adjacent to the nuclear envelope (Fig. 6, 16h). By virtue of their size, frequency, and location within the nucleus we felt these clusters of granules were likely to be the brightly staining peripheral nuclear structures observed by immunofluorescence with anti–snRNP antibodies after HSV infection. Further support for this contention was the finding that these granule clusters were stained by the regressive staining technique of Bernhard (1969), which bleaches chromatin but not ribonucleoprotein (data not shown). This result was suggestive but not proof of the presence of ribonucleoproteins in these nuclear structures. We therefore sought direct evidence for the identity of the immunofluorescent snRNP aggregates and the granule clusters using the techniques of immunoelectron microscopy.

Analysis of snRNP and hnRNP Protein Localization by Electron Micrograph Immunocytochemistry

HSV-infected HEp-2 cells were embedded in Lowicryl K4M and stained using an indirect immunogold procedure with either antibodies specific for either the Sm antigen (Fig. 7, Sm) of snRNP complexes or the chrp polypeptides (Fig. 7, chrp) of hnRNP. The snRNP antigens, while exhibiting some residual component diffusely distributed throughout the nucleus, were intensely stained in the large granule clusters at the periphery of the nucleus (Fig. 7, Sm, arrow). The nucleolus, dense bodies, and cytoplasm did not significantly stain. It was clear that the granule clusters were not nucleolar components altered by the action of the virus on the host nucleus. The snRNP complexes recognized by the Sm-specific antibody are not found in the nucleolus. Furthermore, clearly identifiable nucleolar structures were not stained. Also, the large granule clusters did not stain when sections were treated with an antibody specific for the nucleolar protein B23 (results not shown).

The hnRNP chrp polypeptides were not found in these clusters, but rather appeared to be more concentrated at the periphery of the chromatin regions (Fig. 7, chrp). This possibly indicates the sites of residual host transcriptional activity or transcription complexes collapsed together upon the reorganization of the chromatin after viral infection.

The differences in distribution of hnRNP- and snRNP-
Figure 6. Herpes virus-induced changes in the fine structure of human epidermal HEp-2 cell nuclei. Either mock-infected cells (M) or cells 16 h after infection with HSV-1 (16h) were fixed with glutaraldehyde and osmium tetroxide, then embedded in Epon. Upon infection with HSV much of the chromatin is found at the periphery of the nucleus. Often observed associated with the edges of chromatin are large clusters of granules (gc) not found in uninfected cells. Chromatin (c), dense body (db), granule clusters (gc), nuclear envelope (ne), nucleolus (no), nucleolar remnant (nr), viral particles (v), and dense particles (x). Sections were stained with uranyl acetate and lead citrate. Bar, 0.5 μm.
Figure 7. Localization of nuclear ribonucleoprotein polypeptides in HSV-1-infected human epidermal cells. HEp-2 cells collected 16 h after infection were fixed with PLP and embedded in Lowicryl K4M. Sections were stained with either a mouse monoclonal antibody specific for the Sm antigen of snRNP particles (Sm) or chicken polyclonal antibodies formed against all the chrp polypeptides of hnRNP complexes (chrp). Immunoglobulin binding was visualized by reacting the sections with an appropriate secondary antibody coupled to 15 nm colloidal gold. The large clusters of granules (gc) observed in HSV-infected HEp-2 cells stain heavily with Sm-specific antibodies (Sm), while chrp polypeptides are absent (chrp) and are found diffusely distributed throughout the nucleus with a higher concentration present at the periphery of the chromatin regions. Sections were stained with uranyl acetate. Bar, 0.5 μm.
associated polypeptides was emphasized by the result of double label experiments (Fig. 8). Thin sections of HSV-infected HEp-2 cells were simultaneously stained with chrp and Sm-specific immunoglobulins followed by gold-coupled secondary antibodies. The large granule clusters were heavily stained by the Sm antigen-specific (7-nm gold) antibody while the chrp polypeptides (15-nm gold) were absent. As we will discuss below it is most likely that the granule clusters arise from an enlargement, then relocation and partial condensation, of the interchromatin granule regions of the host cell.

Discussion

Our immunocytochemical localization of host and viral antigens has demonstrated a progressive partitioning of the host cell nucleus during HSV infection. The major components of host cell mRNA transcription and processing complexes are for the most part segregated from the internal nuclear domains which appear to be dominated by viral transcription and replication. Host cell DNA, hnRNP, and snRNP all to some extent become marginalized to the nuclear envelope. However, while the host DNA and hnRNP core proteins appear to colocalize, the snRNP antigens are more concentrated in specific granule clusters which appear to be a characteristic of HSV infection, since a number of other factors interfering with normal nuclear functions did not reproduce the effect.

The localization of the basic hnRNP A and B core proteins (chrp) is of some interest. Infection did not cause the general diffusion of these antigens throughout the nucleus as might have been expected if host RNA polymerase II transcriptional initiation was shut down and transcripts were released from the DNA. As noted in the introduction, the extent and nature of changes wrought in host mRNA transcription by HSV infection remain unclear. As we have shown that chrp polypeptides bind to nascent hnRNA (Martin and Okamura, 1981; Fakan et al., 1986), it seems likely that these proteins remain associated with transcription complexes on the DNA as the chromatin collapses and margrantes during the progression of HSV infection. Our data, however, do not distinguish between a continuation of active transcription at these sites or the presence of arrested transcription complexes as a result of inhibition of chain elongation, or cleavage-polyadenylation and termination. Some hnRNP core proteins remain distributed throughout the nucleus and these could participate in viral transcription. So far, very little is known of the molecular nature of HSV transcription complexes. The chrp proteins have been reported to be associated with SV40 and adenovirus transcripts.

The redistribution of the host cell snRNP antigens is more suggestive and has three aspects. First is failure to localize significantly to regions having the highest concentration of hnRNP antigens. Previous immunofluorescence (Sass and Pederson, 1985; Martin, T. E., U. Scheer, and S. A. Monsma, unpublished results) and immunoelectron microscope studies (Fakan et al., 1984) suggest that a considerable amount of the nuclear snRNP is at transcription sites. Further, we have presented evidence that snRNP antigens in addition to hnRNP proteins are associated with nascent transcripts (Fakan et al., 1986). Structures suggestive of processing complexes, presumed to contain snRNP, have been observed in the region of splice sites in nascent RNP chains (Osheim et al., 1985). Our present data may be taken to suggest that host cell transcripts may be depleted of snRNP during infection. This would be expected to interfere with normal processing events, including splicing, cleavage-polyadenylation, and possibly RNA chain termination (the roles of snRNP were reviewed by Sharp, 1987).

A second aspect of the reorganization of the host cell snRNP complexes is the nature of the granule clusters which enlarge and marginate in the nucleus during HSV-1 infection. It has been known for many years that antibodies to the general U-snRNP Sm antigen and the U-1-snRNP-specific polypeptide give both speckled and diffuse immunocytochemical patterns in mammalian nuclei (Tan, 1979). We have suggested that while the diffuse pattern at least in part represents a colocalization with hnRNP and sites of transcription, the speckles are the light microscope equivalent of the groups of interchromatin granules observed by electron microscopy (Fakan et al., 1984). The fine structure of the granule clusters observed at the periphery of HSV-1-infected nuclei (Figs. 7 and 8) is consistent with these clusters being enlarged and somewhat condensed aggregates of interchromatin granules. The interchromatin granule region may contain an inactive pool of snRNP complexes. We may explain the margination of the snRNP clusters by an apparent physical association with the host chromatin. The redistribution of these host cell nuclear components could result from altered interactions with nuclear matrix structures; an association of viral proteins with nuclear matrix preparations has been described (Bibor-Hardy et al., 1982, 1985; Knipe and Spand, 1982). The question arises therefore as to whether the HSV acts by modification of a host component and/or by synthesis of a virally encoded product to cause host cell snRNP aggregation. As we have described, the few viral proteins so far examined—ICP4, ICP8, ICP5, and p40—do not appear to be present at substantial levels in the aggregates. None of these are likely candidates, however, and others must be sought.

The final aspect of snRNP redistribution which must be considered is the possible requirement of functional snRNP for the proper processing of HSV transcripts. While there appears to be a considerable concentration of the host snRNP in the large granule clusters, there clearly remains some host snRNP distributed throughout the nuclear interior where we presume HSV transcription is occurring. We may note, on the other hand, that few HSV genes contain introns and the

Figure 8. Simultaneous localization of snRNP and hnRNP polypeptides in HSV-1-infected HEp-2 cells. Human epidermal cells were fixed with PLP 16 h after infection. The cells were embedded in Lowicryl K4M. Thin sections were stained with antibodies specific for Sm (7-nm gold) and chrp (15-nm gold) polypeptides. Antibody binding was visualized by staining sections with gold-coupled secondary immunoglobulins. The granule clusters (gc) are heavily stained by the snRNP Sm-specific antibodies. The hnRNP chrp polypeptides are not components of these structures and are found distributed throughout the nucleus. Cytoplasm (c), granule clusters (gc), nucleus (n), nuclear envelope (ne). The sections were stained with uranyl acetate. Bars, 0.5 μm.
early (α) genes account for a number of these. A low level of functional host snRNP may be sufficient, if necessary at all, for the relatively low requirement for HSV pre-mRNA splicing. The need for snRNP in HSV cleavage-polyadenylation has not been addressed, and it is possible such snRNP complexes represent a minor fraction of the total host cell complement.

In conclusion, it seems to us that HSV-specific mechanisms may act to reduce the efficiency of host cell gene expression at the level of pre-mRNA processing by modification of the snRNP complement, thereby increasing the competitive advantage of the virus in the cell. There are several observations suggesting that HSV infection interferes with host cell pre-mRNA processing and mRNA transport. Schek and Bachenheimer (1985) have reported the relative accumulation of a transcript of the γ-actin gene which is larger than the mature mRNA, in HSV-I-infected cells. Furthermore, Bastow et al. (1986) have suggested that HSV-2 can interfere with the maturation or transport of dihydrofolyate reductase pre-mRNA in methotrexate-resistant cells. The role of snRNP in mRNA transport remains speculative, but we anticipate that the further investigation of the state of snRNP in the HSV-infected cell may illuminate both host and viral processes.

We thank Yasmin Orandi, Mary Lynn Parish, and Nanette Solty for the preparation and infection of cultured cells. We also thank Martin Zweig for gifts of monoclonal antibodies.

The different aspects of this research were supported by grants from the Bristol Myers Research and Education and the Leonard S. Florsheim, Jr. Funds, and the Illinois Chapter of the Lupus Foundation of America. General support for the laboratories was provided by United States Public Health Service grants ROI CA12550 (T. E. Martin) and ROI CA21776 (P. G. Spear). The electron microscope facilities were supported by the University of Chicago Cancer Center Core grant CA 14599.

Received for publication 16 March 1987, and in revised form 29 June 1987.

References

Bastow, K. F., J. Bouchard, X.-J. Ren, and Y.-C. Cheng. 1986. Synthesis of dihydrofolate reductase and metabolism of related RNA in a methotrexate-resistant human cell line infected with herpes simplex virus type 2. Virology. 149:199-207.

Barnard, W. 1969. A new staining procedure for electron microscopical cytol-ogy. J. Ultrastruct. Res. 27:250-265.

Beyer, A. L., M. E. Christensen, B. W. Walker, and W. M. LeStourgeon. 1981. Identification and characterization of the packaging proteins of core 40s hnrRN particles. Cell. 11:127-138.

Bibor-Hardy, V., M. Bernard, and R. Sinard. 1983. Nuclear matrix modifications at different stages of infection by herpes simplex virus type 1. J. Gen. Virol. 66:1095-1103.

Bibor-Hardy, V., M. Puchelet, E. St-Pierre, M. Herzberg, and R. Sinard. 1982. The nuclear matrix is involved in herpes simplex virogenesis. Virology. 121:296-306.

Billings, P. R., R. W. Allen, F. C. Jensen, and S. C. Hoch. 1982. Anti-RNP monoclonal antibodies derived from a mouse strain with lupus-like autoimmunity. J. Immunol. 128:1176-1180.

Brown, S. W. 1966. Heterochromatin. Science (Wash. DC). 151:417-425.

DeMey, J., M. Moorzmann, G. Geuens, R. Nuydens, and M. De Brabander. 1981. High resolution light and electron microscopic localization of tubulin with the IGS (Immuno Gold Staining) method. Cell Biol. Int. Rep. 5:889-899.

Dreyfus, G. 1986. Structure and function of nuclear and cytoplasmic ribonucleoprotein particles. Annu. Rev. Cell Biol. 2:459-498.

Fakan, S., G. Leser, and T. E. Martin. 1984. Ultrastructural distribution of nuclear ribonucleoproteins as visualized by immunocytochemistry on thin sections. J. Cell Biol. 98:358-363.

Fakan, S., G. Leser, and T. E. Martin. 1986. Immunoelectron microscope visualization of nuclear ribonucleoprotein antigens within spread transcription complexes. J. Cell Biol. 103:1153-1157.

Fenwick, M. L. 1984. The effects of herpes viruses on cellular macromolecular syntheses. Comp. Virol. 15:289-300.

Geoghegan, W. D., and G. A. Ackerman. 1977. Adsorption of horseradish peroxidase, ovomucoid and anti-immunoglobulin to colloidal gold for the indirect detection of concanavalin A, wheat germ agglutinin and goat anti-human immunoglobulin G on cell surfaces at the electron microscopic level: a new method, theory and application. J. Histochem. Cytochem. 25:1187-1200.

Grazzner, H. G. 1982. Monoclonal antibody to 5-bromo- and 5-iodo-deoxyuridine: a new reagent for detection of DNA replication. Science (Wash. DC). 218:474-475.

Jones, R. E., C. S. Okamura, and T. E. Martin. 1980. Immunofluorescent localization of the proteins of nuclear ribonucleoprotein complexes. J. Cell Biol. 86:235-243.

Knie, D. M., and A. E. Spang. 1982. Definition of a series of stages in the association of two herpesviral proteins with the cell nucleus. J. Virol. 43:314-324.

Kwong, A. D., and N. Frenkel. 1987. Herpes simplex virus infected cells contain functions which destabilize both viral and host mRNAs. Proc. Natl. Acad. Sci. USA. 84:1926-1930.

Lerner, A. E., M. R. Lerner, C. A. Janeway, Jr., and J. Steitz. 1981. Monoclonal antibodies to heterogeneous nuclear RNA-protein complexes. J. Biol. Chem. 259:1827-1833.

Martin, T. E., and C. S. Okamura. 1981. Immunocytochemistry of nuclear hnrRN complexes. In The Cell Nucleus. H. Busch, editor. Academic Press, Inc., New York. IX:119-144.

Martin, T. E., J. M. Pullman, and M. E. McMillan. 1980. Structure and function of nuclear and cytoplasmic ribonucleoprotein complexes. In Cell Biology: A Comprehensive Treatise. D. M. Prescott, and L. Goldstein, editors. Academic Press, Inc., New York. Vol. 8:137-174.

McLean, I. W., and P. K. Nakane. 1974. Periodate-lysine-paraformaldehyde fixative: a new fixative for immunoelectron microscopy. J. Histochem. Cytochem. 22:1077-1083.

Mount, S. M., I. Peterson, M. Hinterberger, A. Karms, and J. A. Steitz. 1983. The U1 small nuclear RNA-protein complex selectively binds a 5' splice site in vitro. Cell. 33:509-518.

Nii, S., C. Morgan, and H. M. Rose. 1968. Electron microscopy of herpes simplex virus. II. Sequence of development. J. Virol. 2:517-536.

Puvion-Dutilleau, F., E. Pichard, and P. Sheldrick. 1985. Appearance of host-specific nuclear proteins in intranuclear "dense bodies" following herpes simplex infection. Eur. J. Cell Biol. 39:458-468.

Quinlan, M. P., L. B. Chen, and D. M. Knipe. 1984. The intranuclear location of a herpes simplex virus DNA-binding protein is determined by the status of viral DNA replication. Cell. 36:857-868.

Rixon, F. J., M. A. Atkinson, and J. Hay. 1983. Intranuclear distribution of herpes simplex virus type 2 DNA synthesis: examination by light and electron microscopy. J. Gen. Virol. 64:2097-2092.

Roizman, B., and W. Patterson. 1985. Herpesvirus and their replication. In Fundamental Virology, B. N. Fields and D. M. Knipe, editors. Raven Press, New York. 607-636.

Sass, H., and T. Pederson. 1984. Transcription-dependent localization of U1 and U2 small nuclear ribonucleoproteins at major sites of gene activity in polytene chromosomes. J. Mol. Biol. 180:911-926.

Schek, N., and S. L. Bachenheimer. 1985. Degradation of cellular mRNAs induced by a virion-associated factor during herpes simplex virus infection of vero cells. J. Virol. 55:601-610.

Schweitz, J., and B. Roizman. 1969. Similarities and differences in the development of laboratory strains and freshly isolated strains of herpes simplex virus in HEP-2 cells: electron microscopy. J. Virol. 4:879-889.

Sharp, P. A. 1987. Splicing of messenger RNA precursors. Science (Wash. DC). 235:766-771.

Showalter, S. D., M. Zweig, and B. Hampar. 1981. Monoclonal antibodies to herpes simplex virus type 1 proteins, including the immediate early protein ICp4. Infect. Immun. 34:694-692.

Slot, W. J., and H. J. Geuze. 1985. A new method for preparing gold probes for multiple-labeling cytochemistry. Eur. J. Cell Biol. 38:87-93.

Tan, E. M. 1979. Autoimmunity to nuclear antigens. Science (Wash. DC). 205:1827-1833.

Williamson, D. H., and D. J. Fennell. 1975. The use of fluorescent DNA-binding agent for detecting and separating yeast mitochondrial DNA. Methods Cell Biol. 12:335-351.