Intracellular Distribution of the La Antigen in CV-1 Cells after Herpes Simplex Virus Type 1 Infection Compared with the Localization of U Small Nuclear Ribonucleoprotein Particles

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SUMMARY

The La antigen is known to associate, at least transiently, with a series of small nuclear and cytoplasmic ribonucleoprotein particles (snRNPs and snRNPs), e.g. U1 and U6 snRNPs. In CV-1 cells a monoclonal antibody (MAb), directed against the La protein (LaB5), immunostained intranuclear speckles. These speckles were found to co-localize with speckles that were stained by MAbs directed against either all U snRNPs or only against U1 snRNPs. Two h after infection of CV-1 cells with herpes simplex virus type 1 (HSV-1) (strain HFEM) the staining of nuclear speckles with the anti-La MAb disappeared and the La protein was found quantitatively in the cytoplasm. In contrast nuclear speckles remained stained with the MAbs against the U snRNPs. Similar results were obtained using HSV-1 strains Lenette or 17 syn ÷ or temperature-sensitive (ts) mutants defective either in DNA synthesis (tsS) or in the immediate early protein (M, 175 K) (tsK). Later in infection the La protein returned to the nucleus. Six h after infection most of the nuclear La protein was found to localize within patchy regions. These areas seem to be related to heterogeneous nuclear RNA transcription and/or processing sites, but not to DNA replication sites.

INTRODUCTION

Sera of patients with autoimmune diseases often contain a variety of sets of anti-nuclear antibodies (reviewed in Sontheimer et al., 1983; Gale & McCarty, 1986). Most familiar are antibodies to the Sm, RNP, La and Ro antigens. These proteins form ribonucleoprotein particles (RNPs) with small RNAs. The Sm antigen associates with all uridine-rich small nuclear RNAs (U snRNAs), with the exception of U3 snRNA, while the RNP antigen, an M, 70K protein, forms an RNP complex only with U1 snRNA. The La protein, an M, 50K protein, was described as a transcription/termination factor of RNA polymerase III (Gottlieb & Steitz, 1987). Moreover, a shuttling of the La protein was observed in uninfected CV-1 cells which depended on the activity of RNA polymerase II (Bachmann et al., 1987, 1989). The La protein binds to a series of small RNAs, including the precursor of tRNAs, 5S RNA and the Ro RNAs (Rinke & Steitz, 1982; Hendrick et al., 1981). In addition, the La protein was found to associate at least partially with U1 and U6 snRNPs (Madore et al., 1984; Kunkel et al., 1986). Antibodies against all U snRNPs or against U1 snRNPs are known to give a speckled staining pattern in immunofluorescence studies (Gale & McCarty, 1986). Similarly, a monoclonal antibody (MAb) to La antigen as well as immunoadsorbed monospecific anti-La antibodies (produced in patients) also stain nuclear speckles in addition to a fine granular nuclear staining (Sontheimer et al., 1983; Bachmann et al., 1986a, 1987, 1989 and unpublished data; Sturgess et al., 1988).
In this paper, we present evidence that the La protein co-localizes with the U snRNP speckles of uninfected CV-1 cells. After infection of cells with herpes simplex virus type 1 (HSV-1), the La protein was separated from the U snRNP speckles and first translocated into the cytoplasm. Later in infection the La protein returned to the nucleus, although it did not reassociate with the U snRNP speckles. In contrast to the U snRNPs the La protein seems to remain functionally active after infection.

**METHODS**

**Materials.** The following materials were used. Fluorescein isothiocyanate (FITC; no. F7250), rhodamine B isothiocyanate (RITC; no. R1755), peroxidase-conjugated anti-mouse IgG (no. A5278) and peroxidase anti-human IgG (no. A6029) were from Sigma, nitrocellulose sheets (BA85, no. 401180) were from Schleicher & Schüll and trypsin and EDTA were from Biochrom K.G.

**Antibodies and sera.** Anti La MAb (La1B5) was prepared as described earlier (Bachmann et al., 1986a). MAbs against the Sm or the RNP antigen were obtained from Professor S. O. Hoch (Billings et al., 1982, 1985). Monospecific anti-La antibodies were prepared as follows. Ten patients' sera containing antibodies against the La protein, as checked by counter-immunoelectrophoresis (not shown), were selected and pooled. Isolated IgGs were passed through a column of Sepharose 4B which was coupled to purified La protein (Bachmann et al., 1986). Bound antibodies were eluted and used as monospecific anti-La antibodies (Bachmann et al., 1986b). Control IgGs (termed Tr-IgGs) were prepared as described earlier (Bachmann et al., 1983).

**Cells and viruses.** CV-1 cells were grown either on coverslips or in flasks at a density of 3 x 10^4 cells/cm^2 (Bachmann et al., 1989). They were infected with HSV-1 strains HFEM, Lenette or 17 [ts] or with temperature-sensitive (ts) mutants at high multiplicity (10 p.f.u./cell) (Heeg et al., 1986; Metzger et al., 1988). The ts mutants were defective in either the immediate early (IE) protein (Mts 175K) (tok) or in DNA synthesis (tsS) (Metzger et al., 1988). The HSV-infected cells were harvested after the incubation periods described in Results.

**Immunofluorescence studies.** CV-1 cells grown on coverslips were washed with P/NaCl, and then fixed with methanol containing 0.02% EGTA (1 h at -20 °C). Fixed cells were rehydrated in P/NaCl and stained with FITC-conjugated anti-La MAb. Stained cells were inspected with a Zeiss Axiophot microscope equipped with epifluorescence optics. Photographs were taken with Kodak 400 Tri-X Pan film. For double immunofluorescence microscopy anti-Sm MAb or anti-RNP MAb was directly labelled with RITC. The labelling of the MAbs was performed as described earlier (Haaajman, 1983).

**PAGE and protein blotting.** PAGE was performed in 10% slab gels in the presence of 0.1% SDS as described by Laemmli (1970). The separated proteins were transferred to nitrocellulose sheets according to the method described by Towbin et al. (1979) with the modifications given by Gramzow et al. (1986). The sheets were incubated with anti-La MAb or monospecific anti-La antibodies; the immunocomplexes that formed were visualized by incubation with a 1:500 dilution of either peroxidase-conjugated anti-mouse IgG or peroxidase-conjugated anti-human IgG using the 4-chloro-1-naphthol/hydrogen peroxide procedure (Nakane, 1968). To rule out non-specific staining, controls with Tr-IgGs were run in parallel, and no staining of protein bands was found in these.

**Preparation of nuclear and cytoplasmic extracts.** Nuclear and cytoplasmic extracts were prepared from uninfected or infected CV-1 cells as follows. After trypsin treatment (5 min, 0.5 mg/ml) 10^6 cells were harvested by centrifugation at 800 g for 5 min, washed with phosphate-buffered saline (PBS) (three times, 15 ml each) and resuspended in 2 ml of PBS. Resuspended cells were homogenized with a Dounce Potter L pestle (20 strokes). Nuclei were pelleted by centrifugation (800 g; 5 min at 4 °C). The supernatant was removed and centrifuged at 6000 g for 10 min at 4 °C and used as 'cytoplasmic extract'. This contained less than 1% of the original amount of DNA (determined according to the procedure described by Kissane & Robins, 1958). Pelleted nuclei were resuspended and further purified by sucrose density gradient centrifugation according to the method of Blobel & Potter (1966). Isolated nuclei were resuspended in 2 ml of PBS containing 1 mM-PMSF, homogenized with a Dounce Potter S pestle (20 strokes) and sonicated (Bachmann et al., 1983). After centrifugation (10000 g; 15 min at 4 °C) the supernatant was used as 'nuclear extract'.

**RESULTS**

**IE, early and late RNAs and proteins**

Roizman (1980) divided the RNAs and proteins of HSV-infected cells into three classes as follows: IE (up to 3.5 h post-infection (p.i.)); early (up to 7 h p.i.) and late (maximum 12 to 18 h p.i.) RNAs and proteins. Formation of polykaryocytes begins 4 h p.i. (Falke, 1972). Soon after viral adsorption the cellular protein synthesis is reduced; this shutoff is caused by a virion-associated component and differs from the virus genome-dependent host shutoff (Daksis et al., 1982, 1985).
Fig. 1. Staining pattern of the anti-La MAb is dependent on the cell type used. Using Vero cells (a) in immunofluorescence microscopic studies the anti-La MAb gave a fine granular, nearly homogeneous nuclear staining, while CV-1 cells (b) showed a speckled-type staining pattern. Bar markers represent 10 μm.

Fig. 2. Alteration of the staining pattern of CV-1 cells after HSV infection at different times p.i.: (a) 0, (b) 1, (c) 2, (d) 4, (e) 6 and (f) 12 h. Bar marker represents 10 μm.

1987). Martin et al. (1987) have recently shown that by 6 h p.i. U snRNPs are separated from heterogeneous nuclear RNPs (hnRNPs). For these reasons we chose the following times p.i. for our studies: 1, 1-5, 2, 4, 6 and 12 h.
Fig. 3. Demonstration by immunoblotting of the translocation of the La protein after HSV infection. Sixty μg of nuclear (odd-numbered lanes) or cytoplasmic (even-numbered lanes) extracts from uninfected cells (lanes 1, 2, 9 and 10) or infected CV-1 cells 2 h p.i. (lanes 3 to 6) and 6 h p.i. (lanes 7 and 8) were separated by gel electrophoresis and blotted to nitrocellulose. Using the anti-La MAb (lanes 1 to 4 and 7 to 10) or a purified monospecific anti-La antibody (lanes 5 and 6) the La protein was detectable in cytoplasmic extracts both in uninfected (lane 2) and infected (lanes 4 and 6) cells. In nuclear extracts the La protein was identified in extracts of uninfected cells (lane 1), while both the anti-La MAb (lane 3) and the anti-La monospecific antibody (lane 5) failed to identify the La protein in nuclear extracts of infected cells 2 h p.i. At 6 h p.i. the La protein was relocated to the nucleus and again detectable in both the nuclear (lane 7) and the cytoplasmic (lane 8) extract. Control antibodies reacted neither with the nuclear (lane 9) nor the cytoplasmic (lane 10) extract.

Alteration of the nucleocytoplasmic distribution of the La protein in herpes simplex virus-infected CV-1 cells

Immunofluorescence studies

Recently we were able to show that the immunofluorescence staining pattern of the La protein depends on the functional state of the cells. If most of the La protein is active in a cell, this cell shows a fine granular nuclear staining (Bachmann et al., 1989 and unpublished data). Most Vero cells give such a staining pattern (Fig. 1a). If the La protein is not used quantitatively, the inactive part is stored in nuclear regions, which appear at the level of immunofluorescence as nuclear speckles or at the level of electron microscopy as clusters of interchromatin granules (Bachmann et al., 1989 and unpublished data; Martin et al., 1987). Consequently at the level of immunofluorescence microscopy, besides the fine granular nuclear staining, such cells show a speckled-type staining pattern. As shown in Fig. 1(b) most CV-1 cells give such a staining pattern. Because we intended to compare the nuclear speckled-type staining of anti-U snRNP antibodies with the staining of the anti-La antibody (see below) we used CV-1 cells for our studies. Isolated monospecific anti-La antibodies give the same staining pattern as the anti-La MAb (Bachmann et al., 1988). In addition, after microinjection of FITC-labelled La protein, CV-1 cells also displayed a speckled staining pattern in vivo. This staining was not affected by the fixation technique used (data not shown). Control IgGs were determined to be unreactive with any nuclear or cytoplasmic structure (data not shown).
Localization of La antigen and U snRNPs

After infection of CV-1 cells with HSV-1 (strain HFEM) the staining pattern of the cells changed substantially (Fig. 2). Compared with uninfected cells the number of speckles strongly decreased after virus infection. On average (n = 40) the number of speckles per cell decreased from 60 ± 15 (time 0) to 6 ± 3 (12 h p.i.). Moreover, during the first 2 h p.i. the staining of nuclear speckles with the anti-La MAb disappeared (Fig. 2a to c). In the subsequent period of infection, the nuclear speckles gradually reappeared. Speckle formation restarted from the nuclear membrane 4 h p.i. (Fig. 2d). At this time 15 ± 5 speckles were present in each nucleus, and at 6 h p.i. most of the nuclear La protein was localized within patchy regions (Fig. 2e) and only a few speckles were detectable with the anti-La MAb. Later in infection, even after polykaryocyte formation (12 h p.i.) these few nuclear speckles were still present. However, the intense staining of the patchy regions was now strongly reduced (Fig. 2f). The same results were obtained using the monospecific anti-La antibodies instead of the anti-La MAb (not shown).

Immunoblotting

The alteration of the immunofluorescence pattern was taken as the first hint of a nucleocytoplasmic exchange of the La protein after HSV infection. To prove this assumption nuclear and cytoplasmic extracts from uninfected and infected CV-1 cells were prepared (see Methods). As shown in Fig. 3 (lanes 1 and 2) the La protein was detectable after immunoblotting of 60 µg of nuclear (lane 1) or cytoplasmic (lane 2) extracts of uninfected CV-1 cells with the anti-La MAb. Using the same amount of nuclear (lane 3) or cytoplasmic (lane 4) extract obtained from CV-1 cells 2 h p.i. with HSV-1 (HFEM) the La protein was identified in the cytoplasmic extract only (lane 4); it was not traceable in the nuclear extract (lane 3). The same result was obtained using monospecific anti-La antibodies instead of the anti-La MAb (lanes 5 and 6). The relocation into the nucleus was also checked by immunoblotting. In agreement with the microscopic data, 6 h p.i. the La protein was again detectable in both the nuclear and the cytoplasmic extracts (lanes 7 and 8). Control antibodies failed to stain any protein species under the experimental conditions used (lanes 9 and 10). Consequently, the observed alteration of the
immunofluorescence staining pattern of CV-1 cells infected with HSV is not the result of an alteration of the epitope recognized by the anti-La MAb, but is caused by an alteration of the nucleocytoplasmic localization of the La protein.

**Double immunofluorescence studies using anti-La MAb and anti-U snRNP antibodies**

Recently, Martin et al. (1987) described a separation of U snRNPs from hnRNPs in HSV-infected Vero cells 4 h p.i. The separated U snRNPs were stored in nuclear speckles, which localized close to the nuclear membrane. Therefore, double immunofluorescence studies were performed using the anti-La MAb and well established MAbs directed against either all U snRNPs (anti-Sm MAb) or U1 snRNPs (anti-RNP MAb) (Billings et al., 1982, 1985). As shown in Fig. 4 most of the nuclear speckles in CV-1 cells that were stained by the anti-La MAb were also stained by anti-U snRNP antibodies. CV-1 cells were double stained with the anti-La MAb (Fig. 4a and c) and with either the anti-Sm MAb (Fig. 4b) or the anti-RNP MAb (Fig. 4d). As shown in Fig. 2(a to c) and also in Fig. 5(a and c) during the first 2 h p.i. the staining of speckles by the anti-La MAb disappeared. However, as shown in Fig. 5(b and d), speckles could still be visualized in these cells by using the anti-Sm MAb (Fig. 5b) or the anti-RNP MAb (Fig. 5d). Consequently the nuclear structures corresponding to the speckles did not disappear; only the La protein had now separated from these areas. As shown in Fig. 2(d and e) and also in Fig. 6(a...
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Fig. 6. Double immunofluorescence studies with HSV-infected CV-1 cells 6 h p.i. The cells were stained with either FITC-labelled anti-La MAb (a and c) or with RITC-labelled MAbs directed against the Sm (b) or the RNP (d) antigen. Most of the nuclear La protein is present within patchy regions and only a few speckles were stained by FITC-labelled anti-La MAb (a and c). Most of these La speckles did not co-localize with the Sm (b) or the RNP (d) antigens. Bar marker represents 10 μm.

Alteration of the nucleocytoplasmic distribution of the La protein in CV-1 cells infected with ts mutants of HSV-1

It is now well established that U snRNPs are involved in splicing of hnRNAs (Parker & Steitz, 1987). However, most hnRNAs of HSV are not spliced. In agreement with the interpretation that the speckles are nuclear storage regions, the U snRNPs associate with the speckles after HSV infection (Martin et al., 1987; this paper). Moreover the La protein, a transcription termination factor, assembles with nuclear speckles after inhibition of transcription in uninfected CV-1- and Vero cells (Bachmann et al., 1987, 1989 and unpublished...
Fig. 7. Alteration of the staining pattern of CV-1 cells after infection with \(ts\) mutants. The images 1 h (a and c) and 5 h (b and d) p.i. are given. The \(ts\) mutants were defective either in 1E 175 K protein (a and b) or in DNA synthesis (c and d). Bar marker represents 10 \(\mu m\).

In contrast to the U snRNPs the La protein dissociated from the nuclear speckles after infection with HSV-1 (Fig. 2, 5 and 7). Consequently the La protein might still be active after infection. To obtain information about the functional role of the La protein after infection, CV-1 cells were infected with HSV-1 (strains HFEM, Lenette or 17 syn\(^*\); the parental strain of the \(ts\) mutants) or with \(ts\) mutants. At 39 °C the \(ts\) mutants that we used are defective either in the 175 K 1E protein (\(tsK\)) or in DNA synthesis (\(tsS\)). Therefore, infected cells were either incubated at 34 °C or 39 °C. CV-1 cells infected with HSV-1 (strains HFEM, Lenette or 17 syn\(^*\)) or with the \(ts\) mutants at 34 °C displayed all the staining patterns and c.p.e. (Fig. 2) found for CV-1 cells infected with HSV-1 (HFEM) at 37 °C (not shown). Similarly CV-1 cells infected with HSV-1 (strains HFEM, Lenette or 17 syn\(^*\)) at 39 °C also had the same altered staining patterns (not shown). Moreover, between 1 and 2 h p.i. the nucleocytoplasmic translocation of the La protein occurred also in cells infected with each \(ts\) mutant at 39 °C (Fig. 7 a and c). However, the c.p.e. and the staining of nuclear patches similar to Fig. 2(e) or Fig. 6(a and c) was found in CV-1 cells infected with the \(tsS\) mutant alone (Fig. 7d). In the case of the \(tsK\) mutant, the La protein was also relocated to the nucleus, but here most of it associated with nuclear speckles (Fig. 7c). After prolonged infection no further alteration of this staining pattern was observed (not shown).
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DISCUSSION

In 1986 we described a MAb against the La protein (Bachmann et al., 1986a). Using HEp-2 or CV-1 cells for immunofluorescence studies this MAb displayed a nuclear speckled-type staining pattern (Bachmann et al., 1986a, 1987, 1988, 1989 and unpublished data). Until now only co-localization studies of the La protein with U snRNP speckles were performed, on which anti-La antibodies give a homogeneous nuclear staining (Nyman et al., 1986). We therefore performed double immunofluorescence studies using CV-1 cells, which were demonstrated to show speckles with both anti-U snRNP MAbs and the anti-La MAb. The La protein was found to co-localize with the U snRNP speckles in uninfected CV-1 cells. However, this co-localization was disturbed after HSV-1 infection. Soon after infection of CV-1 cells with HSV-1 the La protein was exported from the nuclear speckles into the cytoplasm; on returning into the nucleus after prolonged infection it was separate from the U snRNP speckles and found mostly in patchy stained regions. These regions are not related to DNA replication, because they were still stained in CV-1 cells infected with the tsS mutant. On the other hand, this staining pattern looks similar to the localization of hnRNPs in HSV-infected Vero cells (Martin et al., 1987). It is known that certain La RNAs associate with hnRNAs and mRNAs (Jelinek & Leinwand, 1978; Harada & Ikawa, 1979; Harada et al., 1979; Harada & Kato, 1980). Moreover, anti-La antibodies are able to coprecipitate cellular but also some virus-encoded hnRNAs and mRNAs (Hamelin et al., 1986). Recently, we established that a nucleocytoplasmic shuttling of the La protein takes place in uninfected CV-1 cells. This nucleocytoplasmic translocation presumably is the result of a cotransport of La RNPs with mRNAs (Bachmann et al., 1987, 1988, 1989). Therefore, we assume that the patchy stained regions correspond to sites of hnRNA synthesis and/or processing presumably of early mRNAs. Because the nucleocytoplasmic translocation observed in virus-infected cells also occurred in CV-1 cells infected with the ts mutants, it seems likely that HSV infection alters the cellular shuttling of the La protein. The reason for this intracellular translocation of the La protein is unclear. Some of the La RNAs are thought to function in the regulation of translational processes, e.g. in repressing or storing of cellular mRNAs (Lerner & Steitz, 1981). Moreover, the La protein was proposed to be involved in regulation of viral mRNA translation in the poliovirus system (Andrews & Baltimore, 1986). Furthermore the EBER RNAs and the VA RNAs, which are virus-coded La RNAs, are thought to function in regulation of translation (Brunel et al., 1985). Similar small RNAs were also detected in HSV-infected Vero cells (Bachmann et al., 1986c). The unidirectional transport of the La protein from the nucleus into the cytoplasm proceeded during the first 2 h p.i. During this phase, the synthesis of cellular proteins is effectively reduced (Roizman & Furlong, 1974; Dakis et al., 1987). This cellular shutoff is caused by a virus-associated component. Therefore, we speculate that during the first phase of HSV infection the La protein is associated with those regulation factors which are released from the nucleus into the cytoplasm.

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