Herpes simplex virus Us11 protein enhances recovery of protein synthesis and survival in heat shock treated HeLa cells

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Abstract One of the herpes simplex virus type 1 (HSV-1) true late gene products, Us11 protein, is brought into the cell by the infecting virion and may play a role in the virally-induced post-transcriptional control of gene expression. Us11 protein forms large oligomers, exhibits RNA binding features, concentrates into the nucleolus and is able to replace Rex protein in post-transcriptional control of human T-cell leukemia/lymphoma virus type I (HTLV-I) expression. As heat shock drastically alters protein synthesis, and because HSV-1 infection stimulates heat shock protein (Hsp) expression, we analyzed the consequence of heat shock in HeLa cells expressing Us11 alone, either transiently or constitutively. No detectable modification of the overall pattern of protein synthesis was observed in cells growing at normal temperatures, including no induction of Hsp expression or accumulation. However, Us11 protein expression induced an enhanced recovery of protein synthesis after heat shock. Moreover, the level of Us11 protein-mediated protection of protein synthesis was similar to that observed for cells made thermotolerant, but only when submitted to a mild heat shock. Finally, Us11 protein expression induced in cells an enhanced survival to heat shock.

INTRODUCTION

In permissive cells, herpes simplex virus type 1 (HSV-1) infection results in the sequential and coordinated expression of the 78 potential genes of the virus. Infection leads to the inhibition of host protein synthesis starting very early, independently of viral protein synthesis (for review, see Roizman and Sears 1990, 1993). This primary shut-off of host protein synthesis is followed by a secondary shut-off, occurring simultaneously to the expression of early viral genes. Although the mechanisms involved in these inhibitions of protein synthesis are not completely elucidated, they require, for the primary

shut-off at least, UL41 protein which is brought into the cell by the infecting virus (Oroskar and Read 1989; Read and Frenkel 1983; Fenwick and Walker 1978; Sydiskis and Roizman 1967; Schek and Bachenheimer 1985; Zelus et al 1996) and, for the secondary shut-off, synthesis of ICP27, an immediate early protein (Uprichard and Knipe 1996; Hardwicke and Sandri-Goldin 1994; Sandri-Goldin and Hibbard 1996; McGregor et al 1996; Phelan et al 1996). Virally-induced inhibition of host protein synthesis is selective since heat shock proteins (Hsp) and ribosomal proteins are still produced even late in infection (Notarianni and Preston 1982; Estridge et al 1989; Phillips et al 1991; Simonin et al 1997).

The shut-off of protein synthesis also occurs concomitantly to several modifications of the ribosomes. S6 ribosomal protein is phosphorylated during the adsorption step of the virus; four other ribosomal proteins are also phosphorylated after infection; and new phosphorylated

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proteins co-purify with the ribosomes during cell fractionation (Kennedy et al 1981; Massé et al 1990a; 1990b; Simonin et al 1995a). One of them is the product of HSV-1 Us11 late gene. Us11 protein is very basic, highly phosphorylated, and forms large oligomers that may explain why the protein co-purifies with the ribosomes (Roller and Roizman 1992; Diaz et al 1993). Us11 protein is brought into the cell by the infecting virus, migrates to the nuclei and also concentrates in the nucleoli (McLean et al 1987; Puvion-Dutilleul 1987). Beside its specific binding to HSV-1 UL34 mRNA (Roller and Roizman 1991), Us11 protein also interacts with the envelope glycoprotein mRNA of human retroviruses (human T-cell leukaemia/lymphoma virus type-I, HTLV-I; and human immunodeficiency virus type 1, HIV-1) and transactivates their expression through a mechanism shared by Rex and Rev regulatory proteins (Diaz et al 1996). Although the precise function of Us11 protein remains unknown in HSV-1 infected cells, the hypothesis has been made that it is involved in nucleo-cytoplasmic transport of specific mRNA, hence playing a role in the post-transcriptional regulation of gene expression during infection (Diaz et al 1993, 1996).

Heat shock alters cellular protein synthesis (for a review see Parsell and Lindquist 1994) and is able to reactivate the expression of latent HSV-1, *in vivo* (Roizman and Sears 1990; 1993; Sawtell and Thompson 1992). Heat shock also induces the disruption of normal protein synthesis concomitantly to Hsp expression. Hsp are evolutionary conserved proteins, the synthesis of which is induced specifically by exposure to supra-optimal temperatures or to other kinds of stress (Georgopoulos and Welch 1993). Heat shock-induced inhibition of protein synthesis is not a simple consequence of high temperature but is a part of the mechanisms facilitating Hsp gene expression (Lindquist 1986; Lindquist and Craig 1988; Nover 1991). Among the mechanisms proposed to explain the heat-induced modifications of translation are inactivation of eIF-2 and eIF-4F initiation factors (Duncan and Hershey 1984; Panniers et al 1985; Lamphear and Panniers 1991), dephosphorylation of ribosomal protein S6 (Kennedy et al 1984) and the collapse of the intermediate filament network to which are attached many mRNAs (Heine et al 1971; Howe and Hershey 1984).

Because of some common mechanisms involved during HSV-1 infection and heat shock, we examined here the possible involvement of Us11 in regulation of cellular gene expression during heat shock. To this end, the pattern of protein synthesis was analyzed in uninfected HeLa cells but modified for transiently or constitutively expressing Us11. In cells growing at 37°C, Us11 protein did not alter the pattern of protein synthesis, whereas, after heat shock, Us11 expression was correlated with an

enhanced recovery of cellular protein synthesis. In addition, cells expressing Us11 exhibited an increased survival to thermal stress.

MATERIALS AND METHODS

Reagents

Nonidet P-40, Triton X-100, desoxycholic acid, dithioerythritol, Trypan blue, protein A-Sepharose 4B and G418 were obtained from Sigma (St Louis, MO). Cell culture media and complements were obtained from Gibco-BRL (Gaithersburg, MD). The specificity of anti-Us11 antibody has already been described (Diaz et al 1993).

Construction of Us11 expressing vectors

Construction of the Us11 expressing vectors has been described previously (Diaz et al 1993, 1996; Simonin et al 1995b). Briefly, the region of HSV-1 (KOS strain) genome containing Us11 gene was first subcloned into pBluescript II KS+ (Stratagene, La Jolla, CA). In this construct (pG-2), the Us11 coding sequence was under the control of its own late viral promoter. In other constructs, the Us11 coding sequence was placed under the control of either the HSV-1 Us12 immediate early promoter (pHSV-Us11) or the cytomegalovirus immediate early promoter (pCMV-Us11).

Cell culture, transfection, selection of cell lines and heat shock treatments

HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal calf serum (FCS, Gibco-BRL, Gaithersburg, MD) in a humidified atmosphere containing 5% CO₂. Transfection experiments were performed by the calcium-phosphate mediated procedure (Graham and Van Der Eb 1973). For transient expression, exponentially growing HeLa cells were plated at a density of 1.5×10^6 cells/78 cm² one night before transfection with 20 µg of DNA. The DNA precipitate was left for 24 h on the cells which were then washed three times with a serum-free medium. After another incubation of 24 h at 37°C in DMEM containing 10% serum, the cells were trypsinized and replated for further experiments. Efficiency of transfection was estimated in parallel experiments using pCMVβ plasmid that contains the gene encoding β-galactosidase under the control of the cytomegalovirus promoter (Clontech, Palo Alto, CA). Cells expressing β-galactosidase were then monitored by 5-bromo-chloro-3-indolyl β D-galactosidase staining (Lim and Chae 1989). Construction and selection of the two clones stably expressing Us11 (HL5a1 and HL5e6) have already

been described (Simonin et al 1995b). A control cell line (HLNeo 7) was also isolated after transfection with the pSVNeo plasmid alone. Details for clone construction and selection have already been described (Simonin et al 1995b). Heat shock treatments were performed as follows: 20 h before the experiment, cells were plated at a density of 2×10^4 cells/cm² in DMEM supplemented with 25 mM Hepes. Heat shock was then performed by immersing culture dishes into a water bath at 43°C, 44°C or 45°C $\pm$ 0.05°C.

Radiolabeling and gel electrophoresis

Cells were pulse labeled with [³⁵S]methionine (1200 Ci/mmol, Dupont NEN, France) for different periods of time in methionine-free DMEM (ICN, Costa Mesa, CA) containing 10% dialyzed FCS. After heat shock, cells were either immediately labeled or allowed to recover for different periods of time at 37°C before being labeled. Thereafter, cells were rinsed in PBS (137 mM NaCl, 2.7 mM KCl, 8.7 mM Na₂HPO₄, 1.5 mM NaH₂PO₄, 1.5 mM KH₂PO₄) and scraped off the dish. At this point, aliquots were withdrawn for determination of protein concentration and of [³⁵S]methionine incorporation. Cells were lysed in boiling SDS buffer (62.5 mM Tris-HCl, pH 6.8; 1% SDS; 0.1 M dithioerythritol; 10% glycerol and 0.001% bromophenol blue). Labeled proteins were then separated by SDS-PAGE (Laemmli 1970) and detected by autoradiography onto BioMax MR films (Eastman Kodak Co).

Cell fractionation, immunoblotting and immunoprecipitation

For cell fractionation, cells were washed in PBS, scraped from dishes and pelleted for 5 min at 1000 g. The resulting cell pellet was either resuspended in Laemmli SDS buffer and boiled for 5 min (whole cell extract) or lysed at 4°C in a buffer containing 10 mM Tris-HCl, pH 7.4; 1 mM MgCl₂; 0.1 mM EDTA; 10 mM NaCl. The lysates were then clarified for 10 min at 2000 g (pellet). The different subcellular fractions were then resuspended or diluted in SDS sample buffer and boiled. Immunoblots, performed as previously described (Arrigo et al 1988), were probed with Us11 antiserum. The primary antibody was detected with anti-rabbit (Us11) immunoglobulins conjugated to horseradish peroxidase (Amersham Corp., UK). Blots were revealed with an ECL kit (Amersham Corp., UK) and autoradiograms were recorded onto X-Omat LS films (Eastman Kodak Co). For immunoprecipitation under native conditions a constant number of radiolabeled cells was analyzed. Cells were washed in PBS and then lysed in RIPA-buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 1% Nonidet P-40; 0.5% deoxycholic acid and 0.1% SDS). After clarification at 10 000 g for 10 min, anti-Hsp70

serum or preimmune serum was added to the cell lysates. Incubation was for 1 h at 4°C before another incubation of 1 h in the presence of protein A-Sepharose 4B (Sigma, St Louis, MO). Thereafter, the protein-A immunocomplexes were centrifuged for 15 sec at 10 000 g and washed several times with RIPA-buffer. Samples were then heated at 95°C for 3 min in SDS buffer and protein A-Sepharose 4B was removed by centrifugation. The immunoprecipitated proteins were then analyzed by SDS-PAGE and autoradiography.

Immunofluorescence analysis

Cells growing on glass cover slips were fixed with 3.7% formaldehyde in PBS pH 7.0 and cell membranes were permeabilized with 1% Triton X-100. Detection of the primary antibody was performed with goat anti-rabbit antibody coupled to fluorescein isothiocyanate (FITC) (Organon-Technica Cappel, France) at a 1/100 dilution. Cover slips were examined and photographed using the appropriate filters with a Zeiss axioskop photomicroscope using a Planapo 63 $\times$ (1.3 Na) lens. Fluorescent images were recorded onto HP5 Plus (Ilford, UK).

Analysis of cell death and/or survival after heat shock

Cell death was monitored by using the vital dye Trypan blue (Wong et al 1989). Then 20 h before the experiment, about 5×10^5 cells were distributed in 6 well dishes. Cells were either kept at 37°C or exposed for 1 h at either 43°C, 44°C or 45°C. All experiments were in triplicate. Cell death was assessed 24 h after heat shock. Cell survival was also determined by clonogenic colony formation assay. Briefly, 24 h before the experiment, cells were plated at different densities depending on the intensity of the stress (see Results). Following heat shock, cells were incubated at 37°C for 10 additional days. They were then rinsed in PBS, and stained with 2% methylene blue in 50% ethanol. The number of single colonies was then determined.

RESULTS

Expression and localization of Us11 protein in HeLa cells at normal temperature and after heat shock

Transient and constitutive expression of Us11 was analyzed in HeLa cells grown at 37°C by immunoblotting of total cellular proteins (Diaz et al 1993; Simonin et al 1995b). Us11 protein was detected in cells transiently transfected with the pHSV-Us11 vector, containing the Us11 coding sequence under the control of Us12 immediate-early gene promoter of HSV-1. A stronger expression of Us11 was observed in cells transiently transfected

Fig. 1 Analysis of Us11 expression in transfected HeLa cells. (A) Immunoblot analysis of Us11 in HeLa cells transiently transfected with either the control pG-2 (lane 1), or the Us11 expressing pHSV-Us11 (lane 2) or pCMV-Us11 (lane 3) vectors. As described in Materials and Methods, the level of Us11 was analyzed 48 h after transfection by immunoblot analysis of total cellular proteins using anti-Us11 serum. All transfections were performed in parallel and equal amounts of total proteins were loaded on gels. Note that cells transfected with the pCMV-Us11 vector display the highest accumulation of Us11 protein. (B) Immunoblot analysis of HeLa cells constitutively expressing pHSV-Us11 vector (lanes 2 and 3); control cells expressing only the neomycine resistance gene (lane 1). Note that cells constitutively expressing pHSV-Us11 display a weaker accumulation of Us11 than pCMV-Us11 expressing cells. (C–E) Immunofluorescence analysis of Us11 cellular localization in transfected HeLa cells. (C) Transient expression of pCMV-Us11 vector in HeLa cells. (D & E) Constitutive expression of HSV-Us11 in HL5a1 cells. Note in both cases the nucleolar accumulation of Us11. In (C) the percentage of cells expressing Us11 was about 50%. Scale bar = 50 μ m. (F) Analysis of Us11 cellular level and distribution in normal and heat shock treated cells. Cell fractionation was performed in HL5e6 cells expressing Us11 that were either grown at 37°C (lanes 1 and 2) or heat treated for 1 h at 44°C (lanes 3 to 6). Cells were analyzed either immediately after heat shock (lanes 4 and 5) or were allowed to recover at 37°C for 2 h before fractionation (lanes 5 and 6). C, cytoplasmic fraction; N, nuclear fraction. A Western immunoblot probed with anti-Us11 antiserum and revealed with ECL is presented.

with the pCMV-Us11 construct that carries the Us11 coding sequence under the control of the cytomegalovirus promoter (Fig. 1A). In contrast, no detectable expression of Us11 was observed in cells transfected with the pG-2 control vector that contained the Us11 gene with its own late promoter (Fig. 1A). Constitutive expression of Us11 was obtained by co-transfecting HeLa cells with the pHSV-Us11 and the pSVNeo plasmids (Simonin et al 1995b; this study). Clones HL5a1 and HL5e6 displayed an expected expression of Us11, while the control cell line HLNeo7, which was transfected with only the pSVNeo vector, was devoid of Us11 protein (Fig. 1B).

The localization of Us11 protein was next determined in both cells expressing Us11 either transiently or constitutively. As expected, in cells transiently expressing Us11

(Fig. 1C), the anti-Us11 antibody decorates the nucleoli; some fluorescence was also observed in the cytoplasm. In cell lines constitutively expressing Us11 (Fig. 1D and E), a background of nuclear fluorescence was also detected in addition to the nucleolar staining. Therefore, in HeLa cells expressing Us11 either transiently or constitutively, Us11 protein exhibits a localization similar to that observed in HSV-1 infected cells.

To determine whether heat shock could modify simultaneously the level of Us11 protein and its intracellular distribution, an immunoblot analysis was performed after cell fractionation of HeLa cells expressing Us11 constitutively (Fig. 1F). Cells were submitted to a mild heat shock treatment (1 h at 43°C); nuclear and cytosolic fractions were purified from control cells and from heat

Fig. 2 Increased protein synthesis recovery after a mild heat shock in HeLa cells stably expressing US11. Control HLNeo7 and HL5e6 cells that constitutively express Us11, either kept at 37°C (panel A) or heat treated for 1 h at 43°C (panel B), were pulse labeled for 30 min with [³⁵S]methionine. Control HLNeo7 cells (panel B, lanes 2, 4, 6, 8 and 10) and HL5e6 cells expressing Us11 (panel B, lanes 1, 3, 5, 7 and 9) treated at 43°C were either immediately pulse labeled (lanes 1 and 2) or allowed to recover for 60 (lanes 3 and 4), 90 (lanes 5 and 6), 120 (lanes 7 and 8) or 150 (lanes 9 and 10) min before being labeled. Autoradiograms of the SDS-PAGE analysis of the labeled proteins are presented.

shock treated cells either immediately after heat shock or after a recovery of 2 h at 37°C. The same level of Us11 protein was detected in control cells (Fig. 1F, lanes 1 and 2) and in heat treated cells, either immediately after heat shock (lanes 3 and 4) or after the period of recovery (lanes 5 and 6). Furthermore, the heat treatment did not modify the intracellular distribution of Us11 protein, a similar amount of Us11 protein being detected in control or heat treated cells in the nuclear as well as in the cytoplasmic fraction.

Enhanced recovery of protein synthesis after heat shock at 43°C in cells expressing Us11 constitutively

We next investigated whether Us11 protein could modulate the protein synthesis inhibition induced by a moderate heat shock performed at 43°C. The pattern of protein synthesis was analyzed in control HLNeo7 cells, and in

HL5e6 cells which express Us11 constitutively, immediately after 1 h of heat shock at 43°C and during the time of recovery at 37°C up to 150 min (Fig. 2). Constitutive expression of Us11 did not alter the apparent pattern of protein synthesis of control cells grown at 37°C (Fig. 2A). Similarly, immediately after heat shock, inhibition of protein synthesis was similar in control and in Us11-expressing cells (Fig. 2B, lanes 1 and 2) suggesting that Us11 expression does not protect against inhibition of protein synthesis induced by heat shock. However, after 60 min of recovery, the rate of protein synthesis was enhanced in cells expressing Us11 (Fig. 2B, lanes 3 and 4). In these cells, an apparent complete recovery of protein synthesis was observed 120 min after heat shock, whereas such a complete recovery was obtained only after 150 min in cells devoid of Us11. These data indicate that Us11 expression is correlated with an enhanced rate of recovery of protein synthesis after a heat shock performed at 43°C.

Increased recovery of protein synthesis after heat shock at 44°C and 45°C in cells expressing Us11 constitutively

To analyze whether Us11 expression also increased the recovery of protein synthesis after drastic heat treatments, control HLNeo7 cells and HL5e6 cells expressing Us11 constitutively were submitted to heat shock at 44°C or 45°C for 1 h. Cells were then allowed to recover for either 3 or 6 h before they were labeled for 2 h with [³⁵S]methionine. As seen in Figure 3A, panel a, 3 h after the heat stress at 44°C, [³⁵S]methionine incorporation into TCA-precipitated material was less inhibited in cells expressing Us11 than in control cells (40% versus 10%). This phenomenon could still be observed 6 h after the heat stress performed at 44°C for 1 h. Even following the heat stress at 45°C, which completely abrogated [³⁵S]methionine incorporation in control cells for at least 24 h (Fig. 3A, panel b), cells expressing Us11 were still able to incorporate radiolabeled methionine (30%). These results were confirmed by SDS-PAGE analysis of the [³⁵S]-labeled proteins (Fig. 3B). As already emphasized (Fig. 2A), similar patterns of protein synthesis were observed in control cells and in cells expressing Us11 when grown at 37°C (Fig. 3B, lanes 1 and 2). However, after heat shock performed at 44°C, the patterns of protein synthesis obtained for these two cell lines differed drastically. For 3 h after the heat stress many proteins were synthesized by cells expressing Us11, while protein synthesis was still inhibited in control cells (Fig. 3B, lanes 3 and 4). This difference in protein synthesis between the two cell lines was even more remarkable when the heat shock was performed for 1 h at 45°C and when protein synthesis was analyzed after a recovery of 6 h (lanes 7 and 8). In these conditions, protein synthesis was still completely inhibited in control HLNeo7 cells, while efficient in HL5e6 cells that express Us11 constitutively. The recovery of a normal pattern of protein synthesis was observed only in cells expressing Us11 24 h after the heat stress. In addition, the heat tolerance mediated by Us11 expression correlated with a better detection of stress proteins, the synthesis of which was normally induced by heat shock. This is particularly clear for the major stress proteins, Hsp70 and Hsp 90. After heat stress, Hsp70 synthesis was more efficient in cells expressing Us11 than in control cells as shown by SDS-PAGE analysis of the immunoprecipitated proteins (Fig. 3C). Identical results were obtained for other cell lines constitutively expressing Us11 (data not shown). Taken together these results strongly indicate that Us11 expression enhances the recovery of protein synthesis after moderate (43°C) or severe (44–45°C) heat shock treatments, the latter usually leading to irreversible damage in the cells.

Enhanced recovery of protein synthesis in cells expressing Us11 transiently

As shown above, the constitutive expression of Us11 enhanced the rate of protein synthesis recovery after heat shock. The possibility that this phenomenon originated from the continuous presence of Us11 was next investigated. To this aim, the rate of recovery of protein synthesis after heat shock was analyzed in cells that transiently expressed Us11. This was assessed by transiently transfecting HeLa cells with either the control pG-2 or the pCMV-Us11 expression vectors. After transfection (48 h later) cells were exposed to 43°C during 90 or 120 min and then labeled with [³⁵S]-methionine for either 3 (Fig. 4A) or 20 h (Fig. 4B). Proteins were then separated by SDS-PAGE and autoradiographed. Figure 4A shows that after a 90 min heat shock at 43°C followed by a 3 h labeling period, untransfected cells and control cells transfected with pG-2 vector displayed similar protein synthesis patterns, albeit very different from those of cells kept at 37°C. Cells transfected with pCMV-US11 vector exhibited a higher rate of protein synthesis than that of untransfected cells or of cells transfected with pG-2 vector. This higher level of protein synthesis was particularly clear for Hsp70 and Hsp90 as well as for some other non-identified proteins. After 120 min of heat shock, protein synthesis was almost completely inhibited in control cells, whereas cells expressing Us11 still displayed the synthesis of several proteins particularly Hsp70 and Hsp90 (Fig. 4A). Moreover, when the labeling period was 20 h after the heat shock, cells expressing Us11 exhibited a pattern of protein synthesis almost identical to that of cells kept at 37°C, while control cells devoid of Us11 expression still displayed a typical heat shock response (Fig. 4B). These results suggest that Us11 expression, per se, is able to stimulate the recovery of protein synthesis after heat shock.

Comparison of Us11-induced protection of protein synthesis with that induced by thermotolerance

Induction of stress protein synthesis by a moderate heat shock confers a transient state of resistance to heat shock, called thermotolerance (Gerner and Schneider 1975). Thermotolerant cells display both a better protection against the heat-induced shut-off of cell protein synthesis, and a better ability to resume protein synthesis after heat shock (Mizzen and Welch 1988; Georgopoulos and Welch 1993). Therefore, the ability of cells expressing Us11 to recover quickly a high level of protein synthesis after heat shock was compared to that of thermotolerant cells. Figure 5 shows that following the 1 h heat stress at 44°C, protein synthesis was similar in cells expressing Us11 (lane 4) and in thermotolerant cells

Fig. 3 Analysis of protein synthesis recovery after a severe heat shock treatment in HeLa cell lines stably expressing Us11. Control HLNeo7 cells and HL5e6 cells constitutively expressing Us11 were either kept at 37°C or heat shock treated for 1 h at either 44°C or 45°C. Heat shock treated cells were then allowed to recover for different periods of time at 37°C, before being labeled for 2 h with [³⁵S]-methionine. (A) Estimation of [³⁵S]methionine incorporation after heat shock performed at either 44°C (a) or 45°C (b). Solid bars: HLNeo7 cells, hatched bars: HL5e6 cells. Hot-TCA precipitable radioactivity was measured as described in Materials and Methods. Incorporation in untreated cells was standardized to 100%. Each value is averaged and standard deviations are indicated (*n* = 3). (B) SDS-PAGE analysis of the labeled proteins described as in (A). Control HLNeo7 cells are analyzed in lanes 1, 3, 5, 7 and 9 while HL5e6 cells expressing Us11 are in lanes 2, 4, 6, 8 and 10. Lanes 1 and 2: untreated cells. Lanes 3 to 6: 44°C heat shock followed by 3 h (lanes 3, 4) or 6 h (lanes 5, 6) of recovery before labeling. Lanes 7–10: 45°C heat shock followed by 6 h (lane 7, 8) or 24 h (lanes 9, 10) of recovery before labeling. An autoradiogram of the gel is presented. All lanes contained the same amounts of proteins. Positions of Hsp70 and Hsp90 are indicated by arrows. (C) Immunoprecipitation analysis of Hsp70. Samples from the experiment presented in (B) were immunoprecipitated using non-immune (p) and anti-Hsp70 (i) sera as described in Materials and Methods. Autoradiogram of the gel containing the immunoprecipitated proteins is presented. Legend of this figure is identical to that presented in (B).

Fig. 4 Increased protein synthesis recovery in HeLa cells transiently expressing Us11. HeLa cells were transfected with either the control pG-2 or Us11 expressing pCMV-Us11 vectors as described in Materials and Methods. After transfection (48 h later) the percentage of transfected cells was estimated to be more than 40%. Cells were heat shock treated for either 90 or 120 min at 43°C and immediately labeled with [³⁵S]methionine for either 3 or 20 h. In each case, control cells, kept at 37°C were similarly labeled. Total proteins were analyzed by SDS-PAGE and autoradiograms of the gels are presented. (A) Cells labelled for 3 h: Lanes 1–3, untransfected HeLa cells; lanes 4–6, pG-2 transfected cells; lanes 7–9, pCMV-Us11 transfected cells. In lanes 1, 4 and 7, cells kept at 37°C; in lanes 2, 5 and 8, cells were heat shock treated for 90 min at 43°C and in lanes 3, 6 and 9 they were exposed for 120 min at the same temperature. (B) Cells labeled for 20 h: Lanes 1 and 2, pG-2 transfected cells; lanes 3 and 4, pCMV-Us11 transfected cells. Lanes 1 and 3: cells kept at 37°C; lanes 2 and 4: cells heated for 120 min. Arrows indicate positions of Hsp 70 and Hsp 90. Note the enhanced recovery of protein synthesis after heat shock in cells transfected with the pCMV-Us11 vector expressing Us11. In these experiments, more than 40% of the transfected cells expressed Us11 protein (data not shown)

(lane 5). However, after a more drastic heat stress performed at 45°C, protein synthesis in thermotolerant cells was still protected, while that of cells expressing Us11 was strongly inhibited. As expected, upon exposure to 45°C, control cells were devoid of detectable protein synthesis. Similar results were obtained with HL5e6, another cell line expressing Us11 (data not shown). In addition, protein synthesis was not above normal when cells expressing Us11 were made thermotolerant before the drastic stress at 45°C. These results indicate that Us11 expression induces an ability to recover protein synthesis similar to that of thermotolerant cells but only for a moderate heat stress (44°C). However, heat protection induced by Us11 expression differs clearly from that of the heat-induced thermotolerance, because cells expressing Us11 were unable to mimic the protection mediated by thermotolerance when the heat stress was performed at 45°C. Another difference between cells expressing Us11 and thermotolerant cells resides in the level of Hsp70 synthesis. Following the heat stress at

44°C, synthesis of Hsp70 was higher in cells expressing Us11 than in thermotolerant HeLa cells after heat shock (Fig. 5, lanes 4 and 5). A similar decreased induction of Hsp70 synthesis was observed when cells expressing Us11 were made thermotolerant before drastic stress at 45°C.

Enhanced survival to heat shock in HeLa cell lines expressing Us11 constitutively

Since Us11 enhances the resumption of protein synthesis after heat shock, we investigated whether the presence of this protein correlated with an increase of cell survival to thermal stress. The cytotoxicity induced by heat shock was evaluated by staining the cells with Trypan blue and by the ability of cells to form colonies. The percentage of cells stained by Trypan blue was analyzed 24 h after exposing cells to heat shock treatments performed at different temperatures. Figure 6 shows that the percentage of Trypan blue negative cells reflecting the percentage of

Fig. 5 Us11-induced translational resumption is less efficient than that mediated by thermotolerance. Control HLNeo7 cells (lanes 1, 3, 5, 7 and 9) and HL5a1 cells expressing Us11 (lanes 2, 4, 6, 8 and 10) were either kept at 37°C (lanes 1 and 2) or heat shock treated for 1 h at either 44°C (lanes 3–6) or 45°C (lanes 7–10). Heat stressed cells were then allowed to recover for 3 h (44°C treatment) or 6 h (45°C treatment) at 37°C. Labeling was for 2 h with [³⁵S]methionine. The effect of thermotolerance (T) was analyzed in lanes 5, 6, 9, and 10. In this case, cells exposed to 44°C for 1 h were allowed to recover for 14 h at 37°C prior to being exposed to the 44°C and 45°C heat challenges described above. Autoradiogram of the analysis by SDS-PAGE of the labeled proteins is presented. Lanes 1 and 2: untreated cells.

cells still alive, was enhanced by Us11 expression. Increasing the temperature of the heat treatment enhanced the difference in the percentages of relative staining between the two cell lines (20% at 44°C and 35% at 45°C).

A colony-forming ability experiment was also performed to test the survival ability to heat shock of the two different clones that express Us11 (HL5a1 and HL5e6), and of the control cell line (HLNeo7). This was assayed by submitting the different cell lines to 1 h heat shock treatments performed at either 43°C, 44°C or 45°C. Cells were then allowed to grow for an additional 10 days before numbering the surviving colonies. After a 43°C heat challenge, 500 and 1000 cells were plated per dish (Fig. 7, lane A and B, respectively). Control cells (HLNeo7) led to the formation of very few colonies after heat shock, whereas cells expressing Us11 (HL5e6 or HL5a1) yielded a large number of surviving colonies. A similar experiment was performed after heat shock performed at 44°C. In this case, 1000 and 2000 cells were initially plated (Fig. 7 lane C and D, respectively). Here again, cells expressing

Fig. 6 Us11 increases cell survival to heat shock as judged by staining with the vital dye Trypan blue. HL5e6 cells expressing Us11 and control HLNeo7 cells were exposed for 1 h to 43°C, 44°C or 45°C and then allowed to recover for 24 h at 37°C. Control cells were kept at 37°C. Stress-induced cytotoxicity was monitored by the vital dye Trypan blue. The value obtained for control cells kept at 37°C is 100%. Black plots, HL5e6 cells; hatched plots, HLNeo7 cells. Each value represents the percentage of Trypan blue-negative cells and is averaged. Standard deviations are indicated ($n = 3$).

Us11 were far more resistant than the control cell lines after this drastic heat stress. This indicates that Us11 expression strongly enhances cell survival to heat shock by a factor of 2 to 3 as estimated by scoring colonies after heat shock. In the case of a heat stress performed at 45°C, no colony was formed by the control cell line, while the cell lines expressing Us11 were still able to form colonies (data not shown).

DISCUSSION

Our results show that, in HeLa cells, expression of HSV-1 Us11 leads to an enhanced recovery of protein synthesis following heat shock and to an increased resistance of cells to thermal stress. In these cells, Us11 protein shared the same nucleolar localization as that observed in HSV-1 infected cells. Moreover, control experiments showed that, at normal temperature, Us11 expression did not change the apparent pattern of protein synthesis. This confirms that the strong HSV-1 induced-inhibition of cellular mRNA translation is not mediated by Us11 protein, at least when alone.

Following heat shock, which normally induces a strong repression of protein synthesis simultaneously to a stimulation of stress protein expression (reviewed by Georgopoulos and Welch 1993), Us11 protein was found

Fig. 7 Us11 increases cell survival to heat shock as judged by colony formation assays. Colony formation assays were performed following exposure of the control HLNeo7 cells and cells expressing Us11, HL5e6 and HL5a1, for 1 h at either 43°C or 44°C. Then 24 h before the heat shock treatments, cells were replated in 35 mm 6-well dishes at two different dilutions: 5×10^2 (A) and 1×10^3 (B) cells/dish were used in the case of the 43°C experiment, while 1×10^3 (C) and 2×10^3 (D) cells/dish were used for the experiment performed at 44°C. After heat shock, cells were allowed to grow at 37°C for an additional 10 days before being fixed, stained and scored as described in Materials and Methods. Dishes containing the different stained colonies are presented. Note the increased survival of HL5e6 and HL5a1 cells expressing Us11.

to alter the pattern of protein synthesis. Indeed, Us11 expression stimulated the rate of recovery of protein synthesis, but had no apparent effect on the heat-induced inhibition of protein synthesis. Similar observations were recorded in HeLa cells expressing Us11 gene both constitutively or transiently. This suggests that the protective activity of Us11 protein is not a consequence of a cellular adaptation to the continuous presence of the viral protein.

In cells expressing Us11, the enhanced synthesis of Hsp70 and of Hsp90, albeit to a lesser extent for the latter, might be at the origin of the better recovery of protein synthesis after heat shock. Indeed, Hsp70 is a molecular chaperone that promotes the recovery of protein synthesis after heat shock (Liu et al 1992; Parsell and Lindquist 1994) and Hsp90 has been suggested to regulate protein synthesis (Rose et al 1989; Pal et al 1996). One hypothesis could be that after heat shock, Us11 acts as a co-factor that favors cells to enter the recovery pathway which involves Hsp synthesis.

In thermotolerant cells, the deleterious effects of heat shock on protein synthesis are reduced (Gerner and Schneider 1975; Mizzen and Welch 1988). Indeed heat induction of stress protein synthesis before a drastic heat shock which otherwise would be lethal, usually results in significant, although transient, tolerance to this second heat shock. After a moderate heat challenge Us11 protein-mediated protection of protein synthesis is similar to that induced by thermotolerance. However, following a severe heat stress, thermotolerance was clearly more efficient than Us11 protein for protection of protein synthesis. Therefore, for moderate heat stress, Us11 protein is able to compensate the complex biochemical events occurring in thermotolerant cells, including heat-induced Hsp synthesis. After heat shock, Hsp70 is synthesized to a lower level in thermotolerant cells than in cells expressing Us11. This is probably a consequence of the feed-back inhibition of Hsp synthesis observed in cells containing an already high level of Hsp70 (Mizzen and Welch 1988; Parsell and Lindquist 1994).

Synthesis of heat shock proteins which are necessary for recovery to heat shock usually results in an enhanced cellular survival to thermal stress (Georgopoulos and Welch 1993; Morimoto et al 1994). The apparent protective effect of Us11 protein on recovery of protein synthesis prompted us to test the survival to heat shock of cells expressing this protein. Us11 expression is correlated with an enhanced survival of cells to thermal stress. The mechanisms involved in this increased resistance to heat shock are unknown. However, one might speculate that it is related to the recovery of protein synthesis which is stimulated after heat shock.

Our results provide direct evidence for a causal relationship between the presence of Us11 protein and an enhanced recovery of protein synthesis resulting subsequently in a better survival to heat shock. Whether or not Us11 protein protects viral protein synthesis in HSV-1 infected cells submitted to elevated temperatures is an intriguing hypothesis that could be tested by analysis of protein synthesis after infection by virus containing defined mutations in Us11 gene. This is not an easy task to perform because mutations in Us11 will also alter the genetic information contained in other HSV genes localized in different reading frames of the same DNA molecule. However it is already known that HSV-2 strains lacking the genes equivalent to Us12, Us10 and Us11 of HSV-1 are impaired to grow at 37°C and cannot grow at 38.5°C (Brown and Harland 1987).

During heat shock, the molecular mechanisms involved in the alteration of protein synthesis are still unclear. They may include inactivation of the initiation factors involved in recognition of the cap structure at the 5'-end of the mRNAs (Duncan and Hershey 1984), disruption of nucleolar architecture and function (Welch and Suhan 1985), or alteration of the ribosomes such as dephosphorylation of ribosomal protein S6 (Kennedy et al 1984; Duncan 1996). Preliminary experiments, from our laboratories, rule out a possible interference of Us11 with S6 dephosphorylation during heat shock. However, it remains to be demonstrated whether or not Us11 is able to decrease the conversion of eIF2 α and eIF4E into their inactive forms normally observed during heat shock (Duncan 1996). In the nucleolus, Us11 may play a role similar to Hsp70 in protecting ribosome biogenesis (Arrigo et al 1980; Pelham 1984; Pelham et al 1984; Velasquez and Lindquist 1984; Welch and Feramisco 1984). However, whether Us11 acts as a viral chaperone that mimics and/or interacts with Hsp70 is still unknown. Another possible and more probable role of Us11 in heat shock treated cells relates to the recently described ability of this protein to bind specific mRNAs and to promote their translation (Diaz et al 1996). Therefore it is possible that the enhanced recovery of protein synthesis observed after heat shock in cells that express Us11 is due to the

binding of Us11 to specific cellular mRNAs followed by their translation.

Such a protective activity of Us11 is not unique among viral proteins. Recently we have shown that the capsid protein of Semliki Forest Virus (C-protein) also induces thermotolerance of the translational apparatus and thermoprotection in HeLa and monkey CV-1 cells (Michel et al 1994). Both HSV-1 and Semliki Forest Virus share the ability to inhibit cellular protein synthesis soon after infection (Fenwick and Walker 1978; Fenwick and Clark 1982; Van Steeg et al 1984). However, the C-protein and Us11 protein may act differently since the protective activity of the C-protein is dependent of its intracellular concentration, high levels of C-protein inducing a drastic inhibition of translation (Michel et al 1994). Such a behavior was not observed with Us11 protein. Moreover, all virally-encoded proteins that localize in the nuclei after infection do not exhibit such protection to thermal stress. For example, HIV-1 Rev protein is cytotoxic and induces nucleolar dysfunctions (Nosaka et al 1993). This phenomenon is even enhanced after heat shock (Kretz-Remy and Arrigo unpublished observations). Although the functions of Us11 protein and C-protein are probably not identical, they share the intriguing property of helping cells to return to a functional state after exposure to adverse conditions.

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