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Review

Alteration of ribosomal protein maps in herpes simplex virus type 1 infection

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Abstract

At present, the effect of herpes simplex virus infection on the entire proteomes of infected cells is very poorly documented. Following several studies performed over the past few years, the modifications of a sub-cellular fraction induced by herpes simplex virus type 1 can be documented. These studies were performed in order to characterize the virally-induced modifications of a major component of the translational apparatus, the ribosomes. The very basic nature of most of the ribosomal proteins renders them very difficult to separate using isoelectric focusing (IEF). Therefore these studies were achieved using several different but related two-dimensional electrophoretic systems which allowed several two-dimensional ribosomal protein maps to be built. Comparison of the ribosomal protein maps built from non-infected cells with those built from infected cells demonstrated that infection by herpes simplex virus type 1 (HSV-1) induces important modifications of ribosomes: (i) non-reversible phosphorylation of ribosomal protein S6; (ii) unusual phosphorylation of several proteins of the small and the large subunits; and (iii) association of viral and cellular proteins to the ribosomal fraction. An overview of these published studies is presented in this review.

Keywords: Reviews; Ribosomal protein maps; Proteomics; Herpes simplex virus; Poly(A)-binding proteins; Viral proteins

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1. Introduction

The molecular mechanisms governing the interactions between herpes simplex viruses and their eucaryotic host cells remain to be fully elucidated. The tremendous progress made recently in proteomics offers a unique opportunity to point out the numerous changes in host-cells' gene expression induced during productive infection with herpes simplex viruses. However, at present, the effect of herpes simplex virus infection on the entire proteomes of infected cells is very poorly documented. Following several studies performed over the past few years, the modifications of a sub-cellular fraction induced by herpes simplex virus type 1 (HSV-1) can be documented. Indeed, since the end of the 1980s several experiments have been conducted in order to analyze the alterations of ribosomal protein maps of cells infected with HSV-1. These analyses were initially conducted in Madjar's laboratory [1–5] and were continued in this laboratory [6,7]. An overview of these studies and of the techniques used is presented in this review. This introduction summarizes the experimental data reported in the literature before the first experiments and explains the rationale underlying these experiments. The second section details the techniques used for the preparation and analyses of ribosomal proteins, while the final section overviews the most significant published results.

HSV-1 was the first human herpesvirus discovered and is considered a prototype of the alphaherpesvirinae subfamily [8]. Primary infection occurs generally through the oral or genital mucosal tissues by contact of a susceptible seronegative individual with someone excreting HSV-1. A fundamental property of HSV-1 is its ability to become latent after primary infection. During the entire lifetime of the infected individual, the latent virus persists within infected neurons of either trigeminal, sacral or vagal ganglia, in an apparently inactive state and may be reactivated by numerous stimuli. The frequency and the intensity of the reactivation are highly variable from one infected individual to another [9].

During productive infection following either the initial contact with the virus or a reactivation, the physiology and morphology of the infected cells are profoundly modified. Viral genome expression occurs following three sequential steps that are coordinately regulated and leads to the synthesis of very early (α), early (β) and late (γ) proteins. Expression of α genes, controlled by the transcriptional transactivator factor α-TIF brought into the cell by the virions, activates transcription of β genes. Then, both α and some β gene products stimulate the expression of γ genes as well as viral DNA synthesis. Finally, expression of α and β genes is inhibited by late viral proteins. Expression of the viral genome is accompanied by a shut-off of the synthesis of most host proteins that occurs in two stages, a primary shut-off occurring very early after infection and a secondary shut-off occurring simultaneously with the synthesis of β genes (for review see Ref. [10]). Experiments performed since the discovery of the virus, in the first quarter of the last century, up to the late 1980s strongly supported the hypothesis that the virally-induced shut-off, at least the secondary one, resulted in part from translational control [11–14]. For example, it was known that at this time of infection, an increasing amount of non-translating polyribosomes accumulate in infected cells [15]. Remarkably, the virally-induced inhibition of host protein synthesis is selective since several cellular proteins are still produced even late in infection [6].

Other experiments performed during this period demonstrated that another virally-induced modifica-

tion of the host cell concerned nucleoli and more particularly ribosomes. Soon after infection, nucleoli, which are the site of ribosome synthesis, increase in size, localize close to the nuclear membrane and finally become fragmented in small pieces [10]. In addition, the phosphorylation of the ribosomal protein S6 is stimulated by infection [16,17].

Taken together, all the above data suggested that complex regulatory mechanisms involving transcriptional and translational controls take place in infected cells and supported the hypothesis that ribosomes might be sequentially modified during infection. To verify this hypothesis, a first group of experiments was initiated to analyze ribosome and protein synthesis modification of human cells during the course of infection with HSV-1 [5]. These experiments allowed identification of some of the ribosomal proteins which are phosphorylated after infection and determination of their kinetics of phosphorylation [2,5,18]. Other experiments performed since then allowed us to identify all the ribosomal proteins which are phosphorylated during infection, to identify non-ribosomal proteins which appear associated with ribosomes, and finally to demonstrate that synthesis of ribosomal proteins persists late in infection, while that of most of the other cellular proteins is inhibited.

2. Methods of analysis of proteins

The following methods were used for all the experiments reported here.

2.1. Analysis of total proteins

2.1.1. Infection of cells and radioactive labeling of proteins

HeLa cells were plated into Petri dishes and incubated at 37 °C for 16–18 h. Pre-confluent cells were infected with HSV-1, macroplaque strain, at a multiplicity of infection of 20 plaque-forming units (PFU) per cell. The medium (M 199) was supplemented with 1% inactivated fetal calf serum (FCS). After 1 h at 33 °C, the inoculum was replaced by M 199 containing 1% inactivated FCS and the infected cells were incubated at 37 °C for different periods of time. For radioactive labeling, the medium was replaced 1 h before harvesting, by methionine- and cysteine-free minimum essential medium supplemented with 1% inactivated and dialyzed FCS containing a mixture of [^{35}S]methionine and [^{35}S]cysteine at a final concentration of 1.85×10^{6} and 0.925×10^{6} Bq/ml, respectively.

2.1.2. Purification of total proteins

After infection and labeling, the medium was discarded and cells were scraped off and washed twice with ice-cold PBS (130 m*M* NaCl, 4 m*M* $Na_2HPO_4\cdot2H_2O$, 1.5 m*M* KH_2PO_4). Harvested cells were resuspended in 50 m*M* Tris–HCl buffer at pH 7.5 containing 25 m*M* KCl and 5 m*M* $MgCl_2$ (300 μl for 5×10^{6} cells). At this stage, total proteins were extracted using the acetic acid extraction method previously described [19–21]. This method allows a fraction of proteins to be obtained which is highly soluble in isoelectric focusing (IEF) buffer and devoid of nucleic acids. Briefly, the cell suspension was adjusted to 0.2 *M* magnesium using $(CH_3COO)_2Mg\cdot4H_2O$ before addition of 2 vol. of cold glacial acetic acid. After 1 h at 4 °C with occasional vigorous agitation, the precipitated nucleic acids were removed by centrifugation at 7500 *g* at 4 °C for 10 min. The supernatant was kept at 4 °C during the time required for the second extraction of the pellet. For this, the pellet was incubated for 30 min in 200 μl of 0.1 *M* $(CH_3COO)_2Mg\cdot4H_2O$ and 2 vol. of cold glacial acetic acid. The precipitated nucleic acids were removed by centrifugation. Both supernatants were pooled and dialyzed three times against 1000 vol. of 1 *M* acetic acid and stored at −80 °C.

2.1.3. Isoelectric focusing for separation of total proteins

The acetic acid soluble fraction of ^{35}S-labeled proteins was separated as described [6]. Aliquots of each sample containing 20 μg of protein [22] were lyophilized and resuspended in IEF buffer containing 8 *M* urea, 4% CHAPS and 40 m*M* Tris base. The sample was then diluted with 8 vol. of a solution containing 8 *M* urea, 2% CHAPS, 20 m*M* dithioerythritol (DTE), 0.5% (v/v) immobilized pH gradient (IPG) buffer, pH 4–7 (Amersham Pharmacia Biotech). IEF was carried out in the IPGphor™ using linear pH 4–7 IPG DryStrips

(Amersham Pharmacia Biotech). IEF was performed at 20 °C with 40 000 Vh after 14 h of rehydration. At the end of the focusing time, the IPG strips containing focused proteins were equilibrated immediately for 15 min in 5 ml of 50 m*M* Tris–HCl, pH 8.8 containing 6 *M* urea, 30% (v/v) glycerol, 2% (w/v) sodium dodecyl sulfate (SDS), 65 m*M* DTE and traces of bromophenol blue. The strips were then incubated for 15 min in 5 ml of the same solution than that used for equilibration except that the DTE was replaced by 135 m*M* iodoacetamide. At the end of the incubation times, the strips were loaded onto a 1 mm thick 12.5% (w/v) polyacrylamide slab gel. The second dimension was performed at 20 °C in the presence of SDS with 650 Vh [23]. Proteins were visualized by silver staining of the gels [24]. Gels were dried and submitted to autoradiography.

2.2. Analysis of ribosomal proteins

The method used to purify ribosomes and ribosomal proteins allows a mixture of total ribosomal proteins (TP80S) free of RNA molecules to be prepared. This procedure originally developed by Fraenkel-Conrat [19] was modified and described in great detail in Refs. [20,25,26]. It permits the subsequent separation and analysis of ribosomal proteins by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE).

2.2.1. Purification of ribosomes

Ribosomes were purified after infection and/or ^{35}S-labeling. For this, 15×10^6 mock-infected and HSV-1-infected HeLa cells were washed three times with cold PBS, kept at 4 °C, and scraped off the flask in 10 ml of PBS. After centrifugation of the cell suspension at 500 *g* for 5 min at 4 °C, cells were resuspended in 0.3 ml of cold 0.25 *M* sucrose, 25 m*M* KCl, 5 m*M* $MgCl_2$, 50 m*M* Tris–HCl, pH 7.4 (buffer A), containing 0.7% NP-40, and left on ice for 10 min to disrupt the cell membranes. Nuclei were then spun down by centrifugation at 750 *g* for 10 min at 4 °C and removed. The postnuclear supernatant was centrifuged at 12 500 *g* for 10 min at 4 °C to spin down the mitochondria. The KCl concentration of the postmitochondrial supernatant was precisely adjusted to 0.5 M.

The postmitochondrial supernatant was layered on top of 1 ml of sucrose cushion made of 1 *M* sucrose, 0.5 *M* KCl, 5 m*M* $MgCl_2$, 50 m*M* Tris–HCl, pH 7.4. After centrifugation for 2 h at 245 000 *g* at 4 °C, the supernatant was removed from the pellet of ribosomes. The ribosome pellet was quickly washed twice with cold water, then resuspended in 300 μl of buffer A. The amount of ribosomes was estimated by measuring the optical density at 260 nm, assuming that 14 A_{260} units correspond to ~1 mg of ribosomes and 0.5 mg of total ribosomal proteins [26].

2.2.2. Extraction, alkylation and lyophilization of ribosomal proteins

Proteins were first extracted by the acetic acid procedure as described above. Then, all the SH groups of the proteins were blocked without change of the net charge of the proteins by reduction by DTE and alkylation by iodoacetamide according to Madjar and Traut [27]. Briefly, the dried proteins were resuspended in 6 *M* guanidine hydrochloride, 0.5 *M* Tris–HCl, pH 8.5, and 10 m*M* DTE at room temperature under nitrogen for 30 min, then alkylated in the presence of 0.04 *M* iodoacetamide under nitrogen for 1.5 h. The mixture was dialyzed against 1 *M* acetic acid to eliminate contaminating salts and proteins were lyophilized in a SpeedVac concentrator [26].

2.2.3. Separation of ribosomal proteins by 2D-PAGE

Except for Sa, S12, P0, P1 and P2, the mammalian eucaryotic ribosomal proteins are very basic. Their p*I* range from 8.83 to 13.46 for rat S21 and L41, respectively [28]. This intrinsic property means it is not easy to analyse them by IEF. However, after many attempts to obtain a reproducible separation of very basic proteins by IEF, Gorg and colleagues [29] have been able to separate under steady state conditions ribosomal proteins of HeLa cells, revealing highly reproducible 2-D patterns. Unfortunately, these systems can not yet be routinely used since the identities of the ribosomal proteins visible in these 2-D patterns are still not known.

Several 2-D electrophoretic systems, dedicated to the separation of ribosomal proteins, have been developed which allow the reproducible separation of all procaryotic as well as eucaryotic ribosomal proteins. The first systems were developed by

Kaltschmidt and Wittmann [30] for separation of *Escherichia coli* ribosomal proteins. Since these pioneer analyses of ribosomal proteins by 2-D PAGE, numerous studies have been carried out using different electrophoretic systems. From these studies, reviewed in Refs. [31,32], a uniform nomenclature for mammalian ribosomal proteins has been proposed [31]. In the same period, Madjar and his colleagues developed a procedure called the ''method of four corner'' [32]. This method, which involved the separation of proteins in four different but related 2-D electrophoretic systems, for the first time allowed the identification of all the ribosomal proteins, without their individual separation and purification. In addition, this method presented the advantages of identifying each of the ribosomal proteins according to the uniform nomenclature and establishing a correlation between the position of one given protein in one of the four gels with that of the same protein in other gel systems used previously [21]. The description and some of the applications of this method have been reviewed previously [33].

The four systems are: system I (acidic–SDS), system II (basic–SDS), system III (basic–acidic), and system IV (acidic–acidic). In brief, the first dimensional separation is performed in identical conditions for systems I and IV, and for systems II and III. It is carried out in tube gels containing 4% (w/v) polyacrylamide and 8 *M* urea at pH 5.5 for systems I and IV or at pH 8.6 for systems II and III. The second dimensional separation is performed in identical conditions for systems I and II and for systems III and IV. It is carried out in slab gels containing 6 *M* urea in either 12.5% (w/v) polyacrylamide at pH 6.75 in the presence of SDS (systems I and II) or in 18% (w/v) polyacrylamide at pH 4.5 in the absence of SDS (systems III and IV).

After electrophoresis and staining, the arrangement of the gels is crucial for identification of the proteins. The top of the first-dimension gels of system I and IV and of system II and III are aligned on the same vertical. Similarly, the top of the second-dimension gels of systems I and II and of III and IV are aligned on the same horizontal. Using such alignments, a given protein is positioned on the same horizontal line in systems I and II, and in systems III and IV while being positioned on the same vertical line in systems I and IV and in systems II and III. Therefore, a rectangle can be drawn, each corner of which corresponds to the position of the same protein [32].

Systems I and IV are used for their ability in resolving acidic proteins that do not enter the first dimension in systems II and III. In systems I and IV, the different phosphorylated and non-phosphorylated forms of a basic protein are in the same spot. On the other hand, in systems II and III, the different phosphorylated forms of a basic protein can be separated. This could allow the determination of the number of phosphate residues incorporated per phosphorylated ribosomal protein [34,35]. Several ribosomal protein maps have been established using these four systems [21,27,32,36,37]. A map of human ribosomal proteins obtained in this laboratory using system II is presented in Fig. 1. At present, the sequences of 78 and 77 ribosomal proteins have been determined from rat and human, respectively. As reported in great detail in Ref. [28], these sequences have been determined either by direct sequencing of the proteins or deduced from the sequences of nucleotides in cDNAs. A compilation of the primary structure of the ~80 mammalian ribosomal proteins has been reported [28]. The electrophoretic gel systems used for most of these identifications were very similar, if not identical, to that presented in Fig. 1 (see for example identification of rat ribosomal protein L10 in Ref. [38]). However, sequencing of all the proteins visible on Fig. 1 has not yet been achieved.

3. Modifications of ribosomal protein maps after HSV-1 infection

The HSV-1 induced alterations of ribosomal protein maps were analyzed in order to characterize the modifications of the ribosomes during the course of infection. These analyses were performed using the four corner method. However, for clarity and because the electrophoretic system II by itself allows a very efficient separation of all the basic ribosomal proteins, only the analyses carried out in system II are shown in this report. An example of a map obtained in our laboratory with ribosomal proteins purified from HSV-1 infected human cells and separated in system II is presented in Fig. 2.

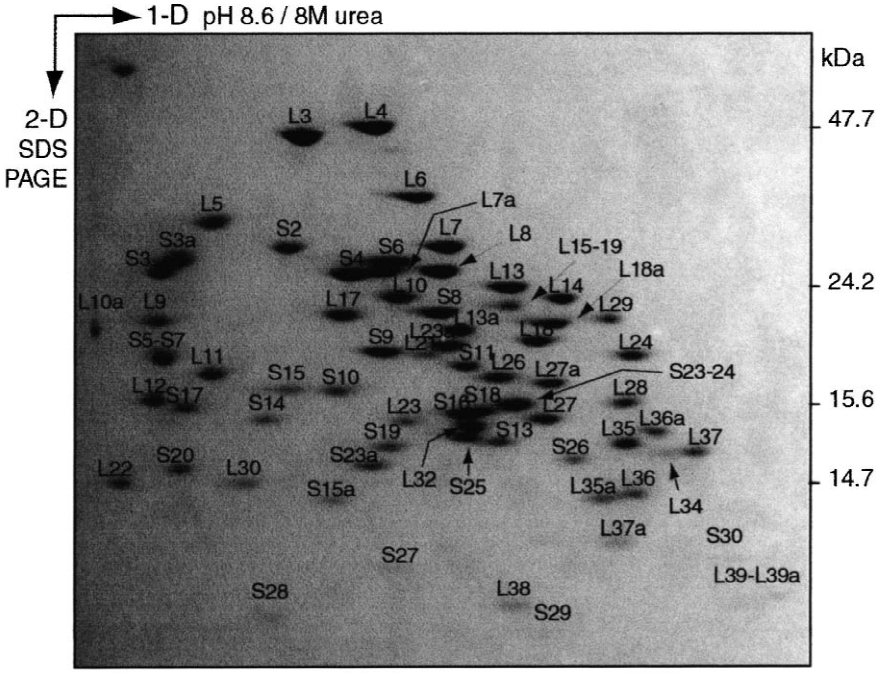

Fig. 1. Two-dimensional ribosomal protein map of basic ribosomal proteins purified from HeLa cells. An amount corresponding to 2.5 A_{260} units of ribosomes was separated in the two-dimensional electrophoretic system II. The first dimension was run at constant voltage (72 V) during 16 h, from the anode to the cathode, in 1.25-mm inside diameter tube gels. The second dimension was performed at 20 °C with 1000 Vh using a maximum power of 6 W per gel. At the end of the electrophoresis ribosomal proteins were visualized by Coomassie brilliant blue staining and numbered according to the uniformed nomenclature [31]. L and S stand for proteins belonging to the large and the small ribosomal subunit, respectively.

3.1. Identification of phosphorylated ribosomal proteins

At the beginning of the study performed by Massé and his colleagues [5], it was not known whether the phosphorylation of ribosomal proteins other than S6 could be stimulated after infection by HSV-1. Therefore to identify these proteins, the following experimental strategy was developed. Mock-infected and HSV-1 human infected cells were labelled with ^{32}P for 90 min at different times after infection. Some of these experiments were carried out after a deprivation of serum because S6 phosphorylation was known to be stimulated by addition of serum [35]. Ribosomes were purified from mock-infected and infected cells and ribosomal proteins were analyzed by the four corner method described above. An example of an HSV-1-modified ribosomal protein map similar to those obtained in the above study is presented in Fig. 2. All together, these experiments demonstrated that HSV-1 induces the phosphorylation of five ribosomal proteins. Four of them, S2, Sa, S3a and S6, belong to the 40S subunit and only one, L30, belongs to the 60S subunit. Using the four corner method, identification of the phosphorylated forms of S2, Sa, S3a and S6 was unambiguous. However, identification of the phosphorylated form of L30, initially designated v2 [5], required the further following experiments [2]. Ribosomal proteins from infected cells were separated in system II and transferred to polyvinylidene difluoride (PVDF) membranes. Ribosomal protein L30 and its suspected phosphorylated derivative (L30a, Fig. 2) were subjected to an in situ CNBr cleavage [39]. The hydrolysis products were eluted and separated through high-resolution discontinuous SDS–PAGE

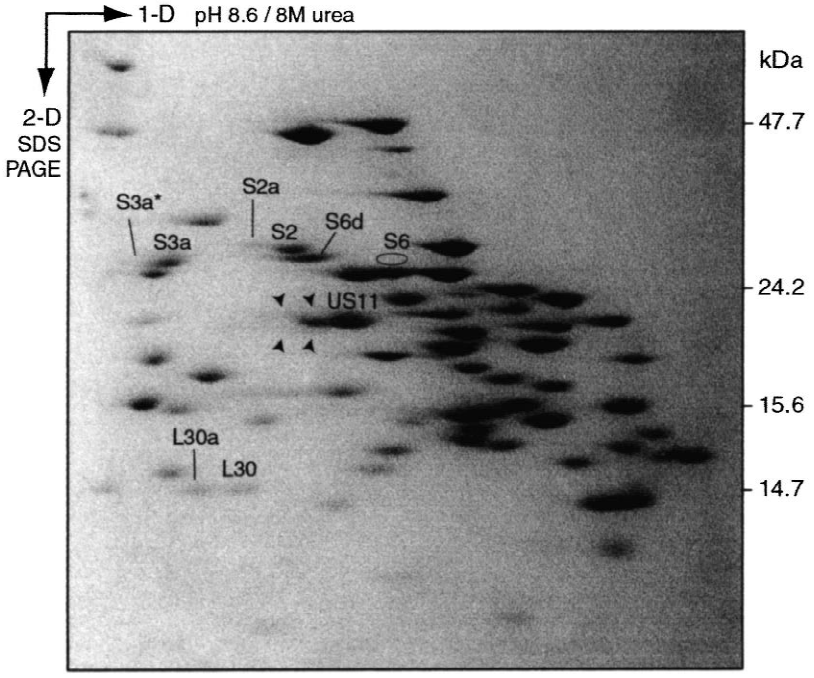

Fig. 2. Alterations of the two-dimensional ribosomal protein map of basic ribosomal proteins in HSV-1 infected cells. HeLa cells were infected for 15 h with HSV-1 (20 PFU per cell). Proteins were extracted from the ribosomal fraction, and 100 μg were separated in system II as indicated in the legend to Fig. 1. For clarity, only the alterations are highlighted. The phosphorylated forms are indicated by a mark to the left of the corresponding proteins. S6 is only present in its fully phosphorylated derivative S6d. S2a, S3a* and L30a are the phosphorylated derivatives of S2, S3a and L30, respectively. US11 viral protein migrates under L17 protein, and its different phosphorylated derivatives marked by arrowheads on its left.

[40] and then transferred to another PVDF membrane. N-terminal aminoacid sequences of the major peptides were determined and compared. This allowed it to be demonstrated that L30a was a derivative of L30. In addition, it was demonstrated that L30a was a phosphorylated form of L30 containing only phosphoserine. This was performed by determination of the phospho-aminoacids of L30a by vapor-phase acid hydrolysis of ^{32}P-labelled L30 immobilized on PVDF membrane [41]. During the course of these experiments, the phospho-aminoacid composition of S2a, S3a* and S6 was also analyzed (Fig. 2). Phospho-serine was the main phospho-aminoacid present in these three proteins, although very small amounts of phospho-threonine were also detected.

Moreover, analysis of the kinetics of phosphorylation of these ribosomal proteins during the course of infection revealed that S6 phosphorylation increases immediately after infection, even in absence of serum, while de novo phosphorylation of S2, S3a, Sa and L30 appear later [2,5–7,18].

3.2. Identification of non-ribosomal proteins associated with ribosomes

From the very start of the analyses it has been observed that non-ribosomal proteins were found in the ribosomal fractions of infected cells. Initially, three proteins named v1, v2 and v3 were pointed out [5]. Later, other proteins were detected and designated protein 1 to protein 7 [7]. These proteins were identified (Fig. 3D).

For identification of v1, total proteins from the ribosomal fraction were extracted by the acetic acid procedure and separated in system II. Proteins were

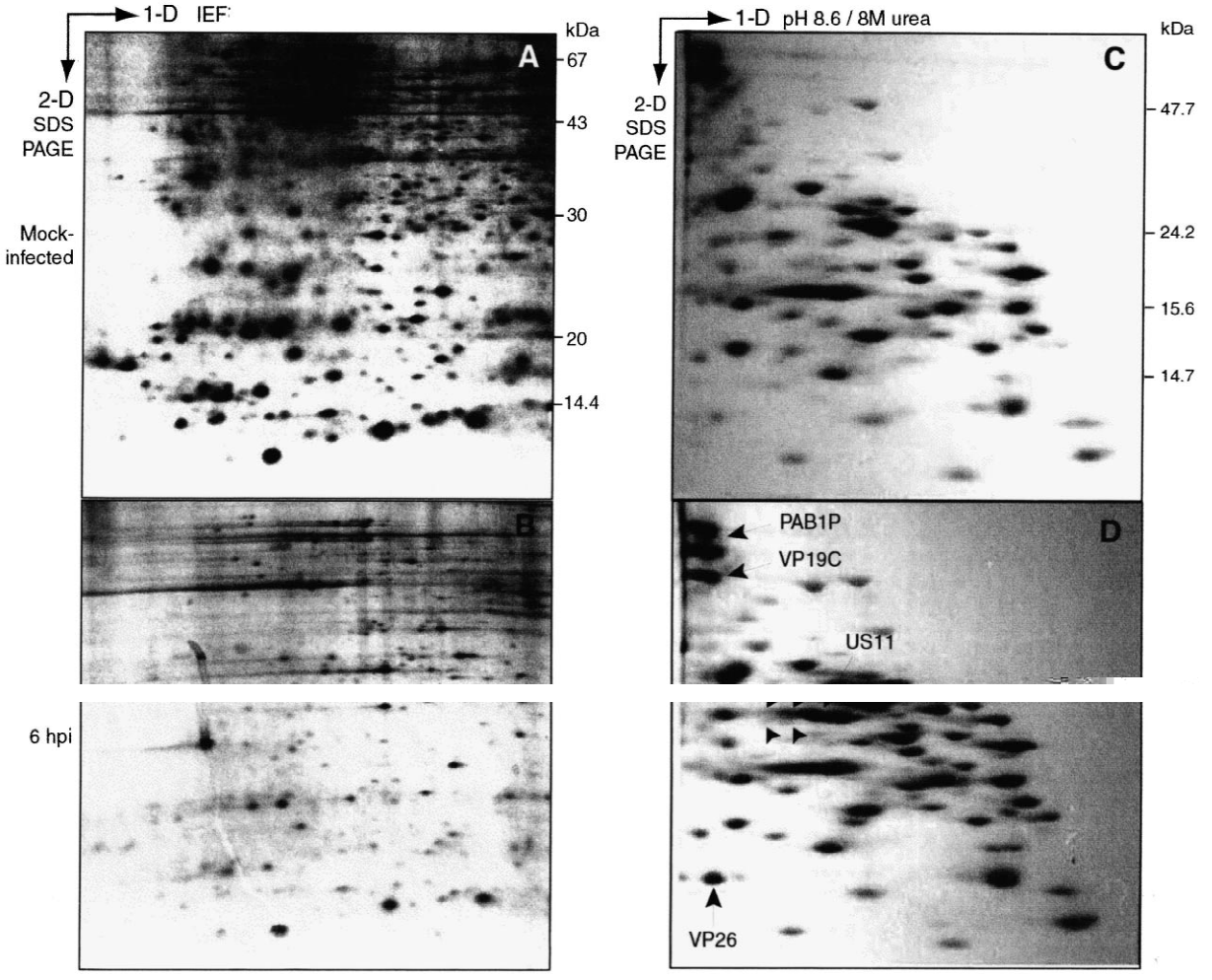

Fig. 3. 2D-PAGE analysis of soluble acidic proteins and basic ribosomal proteins extracted from mock-infected and HSV-1-infected HeLa cells. HeLa cells were infected with HSV-1 (20 PFU per cell) or mock-infected for 6 h. Total proteins (A and B) or proteins from the ribosomal fraction (C and D) were analyzed by 2D-PAGE. Then 5 h after infection, cells were incubated with a mixture of [^{35}S]methionine and [^{35}S]cysteine for one h just before harvesting. Total acetic acid soluble proteins and proteins from the ribosomal fraction were purified and separated. For total proteins, the first separation was performed using a linear pH 4–7 IPG, and the second separation was performed using the Laemmli–SDS system. Separation of proteins of the ribosomal fraction was performed in system II as described in the legend to Fig. 1. At the end of the electrophoreses, acetic acid soluble proteins were visualized by silver staining, and ribosomal proteins by Coomassie brilliant blue staining, then the gels were dried and submitted to autoradiography. A and C, mock infected cells; B and D, 6 h infected cells. The identified non-ribosomal proteins associated with ribosomes in infected cells are indicated by their names in panel D. Any of the proteins separated in A and B have been identified. Molecular weight markers, in kDa, are indicated to the right of panels A and C.

then transferred to PVDF membranes and stained with amido black. As for identification of L30, membrane pieces containing v1 were cut out and pooled for in situ CNBr cleavage. The hydrolysis products were eluted and separated through a high-resolution discontinuous SDS–PAGE then transferred to another PVDF membrane. N-terminal amino-acid sequences of the major peptides allowed it to be determined that v1 was the product of the US11 gene product of HSV-1.

Identification of proteins 1–7 was carried out by N-terminal sequencing and also by peptide mass

determination by mass spectrometry. For this, ribosomal proteins were purified from mock-infected and HSV-1 infected cells and separated in system II. Non-ribosomal protein 1 was present in mock- as well as in infected cells. Proteins 2–7 which were present only after HSV-1 infection might be either viral proteins or cellular proteins whose presence in the ribosomal fraction is observed only upon infection.

Protein 2 was identified by determination of its N-terminal sequence. For this, proteins were transferred to a PVDF membrane after separation by two-dimensional electrophoresis (2-DE). Immobilized proteins were visualized with 0.1% amido black solubilized in 45% methanol and 9% acetic acid, and membrane-bound protein 2 was cut out of the PVDF membrane. N-terminal sequence determination was performed directly by Edman degradation using a gas-phase sequencer, and the protein was identified by comparison with sequences available in different databases. The N-terminal sequence of protein 2, MKTNPLP, matches that of HSV-1 protein VP19C, a component of the viral capsid (SWISS-PROT entry P32888). Its theoretical p*I* is 9.1 and its molecular mass is 50.2 kDa, fitting that estimated for protein 2 (50 kDa) (Fig. 3D).

For identification of protein 1 and proteins 3–7, fragments of the gel containing these proteins were excised from the gel and then digested with trypsin. Extracted peptides from digested proteins were layered on the mass spectrometer target. Identical volumes of solvent (10 mg/ml of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 0.1% (v/v) trifluoracetic acid) were added to the previously loaded digests, and samples were dried as quickly as possible and analyzed by mass spectrometry using MALDI–TOF mass spectrometers (Voyager™ Elite or super STR, PerSeptive Biosystems, Framingham MA, USA). Masses of the major peptides were chosen for protein identification using SmartIdent software. The research was conducted against SWISS-PROT and TrEMBL data bases. The query was made for human and virus proteins.

Peptide mass fingerprinting of protein 1 revealed that this protein should be one of the two poly(A)-binding protein, PABP1 or PABP2. With this analysis alone, even using different techniques, we were not able to conclude whether protein 1 was PAB1P or PABP2. Nevertheless, from other experiments it has been possible to conclude that protein 1 should be PABP1 rather than PABP2 (Fig. 3D).

Peptides obtained for protein 3 (v3) were matched against the SWISS-PROT data base. This, combined with other techniques (Bienvenut et al., in preparation) allowed us to identify this protein as VP26 from HSV-1 (SWISS-PROT entry P10219) (Fig. 3D).

Peptide mass fingerprinting of proteins 5–7 showed that these proteins were phosphorylated derivatives of US11 protein (SWISS-PROT entry P56958) (Figs. 2 and 3D). Peptide mass fingerprinting of protein 4 did not correspond to any known protein.

Because VP19C and VP26 are components of the capsid, and because US11 protein resides in the tegument, one can not exclude that viral particles were co-purified with ribosomes during the cell fractionation procedure. However, this does not seem to be likely. Indeed, these proteins were found associated to the ribosomes as quickly as 6 h post infection and recent data have clearly demonstrated that capsids appear in the cytoplasm only at a very late time of infection [42,43]. Furthermore, VP19C, VP26 and US11 protein were found to associate to ribosomes with different kinetics. All together, these observations argue against a possible contamination of the ribosomal fraction by the viral capsid at this time of infection. However, the role, if any, played by VP19C and VP26 on the translational apparatus, remains to be elucidated.

3.3. Functions of phosphorylated ribosomal proteins

At present, sequences of almost all mammalian ribosomal proteins are known [28]. However, except maybe for S6, their structures and functions within ribosomes remain poorly documented. This is particularly true for Sa whose function is unknown. Not much is known either about the function of S2, although it appears that S2 could be involved in the P site organization of the ribosomal 40S subunit [44,45].

Nothing is known about the function of mammalian ribosomal protein L30. However it is clearly demonstrated that L32, the homologous ribosomal protein in yeast, is involved in the regulation of the

splicing of its own pre-mRNA [46–48]. It is tempting to speculate that mammalian ribosomal protein L30 might play a similar role. If this were the case, its HSV-1 induced phosphorylation could be involved in the modulation of this function, thus regulating in some way the ribosome assembly after HSV-1 infection.

As stated above, the function of ribosomal protein S6 is now quite well elucidated. S6 plays a crucial role in the control of the translation of mRNAs coding for many components of the translational apparatus [49]. Nevertheless, at present, the molecular bases supporting this regulation are not elucidated.

Several ribosomal proteins are bifunctional [50]. Among them, S3a has been shown recently to play a role in cell death and transformation. Constitutive or transient enhanced expression of S3a is considered as a ''priming'' for cells' apoptosis and suppression of such enhanced expression as an ''execution'' [51]. Because HSV-1 modifies the host-cells' proliferation and death programs through an unknown mechanism, one may postulate that HSV-1 induced phosphorylation of S3a participates in this mechanism. It is not known however whether the functions of S3a are regulated by phosphorylation.

3.4. Functions of non-ribosomal proteins associated with ribosomes

3.4.1. US11 protein

Although the exact function of US11 is not fully understood, multiple properties of the protein have been demonstrated suggesting that this protein is plurifunctional and is involved in post-transcriptional regulation of gene expression. As a late gene product, US11 protein is among the most abundant viral proteins present in cells late in infection and is packaged in the tegument of the native virion to be delivered into cells after infection [4,52,53]. Soon after infection, US11 protein of the parental virus delivered into the cell could reduce the accumulation of the activated cellular PKR and sustain protein synthesis. Conversely, US11 protein made late in infection does not block PKR activation. The mechanism by which US11 modulates PKR activity is not known. However, it is probable that functional interferences between the two proteins is correlated with their ability to bind both dsRNA and ribosomes [54,55]. Moreover, it has been shown that US11 protein is able to activate the correct nucleo-cytoplasmic transport and translation of specific mRNAs [56]. From all these observations, it is clear that the intracellular movements of US11 and its association with ribosomes are determinants for its function.

3.4.2. VP19C, VP26

At least 39 of the 80 viral genes encode proteins that are components of the HSV-1 virion which consists of a trilaminar lipid envelope, an amorphous layer tegument, an icosahedral capsid shell, and a DNA-containing core. Capsids are assembled in the nucleus of infected cells, where they are packaged with viral DNA. The major components of the HSV-1 capsid shell are the viral proteins VP5, VP19C, VP23 and VP26 organized into 162 capsomers. The capsomers are made of VP5, the more abundant protein which constitutes the structural subunit, and of the three less abundant proteins, VP19C, VP23 and VP26. VP19C and VP23 are located in the space between the capsomers whereas VP26 sits at the tip of each copy of VP5 and may be involved in linking the capsid to the surrounding tegument and envelope at a late stage of viral assembly (for review see Ref. [10]).

3.4.3. PABP1, PABP2

The poly(A)-binding protein (PABP) is an RNA binding protein which has been identified in mammals, yeast and plants. In human, two PABP (PABP1 and PABP2: SWISS-PROT entries P11940 and Q15097, respectively) have been isolated which bind the poly(A) tail at the 3′ terminus of mRNA [57]. Both proteins are very close in sequence but differ in length. PABP1 is longer than PABP2 and possesses four RNA recognition motifs (RRM) whereas PABP2 possesses only one motif [58,59].

PABP1 has been extensively studied and it is now well established that this protein plays a major role in mRNA turn-over as well as in regulation of translation [60]. By interacting with one of the translational eucaryotic initiation factors (eIF4G), PABP1 allows the 5′-m^7GpppN cap and the 3′ poly(A) tail of eucaryotic mRNAs to communicate and promotes translation initiation. A model has

been proposed in which interaction of PABP1 with eIF4G induces the circularization of mRNAs [61]. This model has been confirmed using atomic force microscopy [62].

In contrast to PABP1, PABP2 interacts slightly with the poly(A) tail of mRNAs and does not interact significantly with eIF4G [58,59,63]. The exact function of PABP2 is still not fully determined, however, it has been proposed very recently that PABP2 could be a component of a so-called ''Pioneer initiation complex''. This complex, which is probably involved only in the first round of translation, might act as a quality control mechanism to eliminate mRNAs which prematurely terminate translation [64].

PABPs are thus multifunctional proteins which participate in the regulation of translation initiation, translation accuracy, degradation and/or stability of mRNA.

3.5. Persistence of ribosomal protein synthesis after infection

Synthesis of ribosomal proteins was analyzed in cells infected with HSV-1 under different experimental conditions [1–3,6]. For this, the HSV-1 induced alterations of ^{35}S-labelled ribosomal protein maps were analyzed during the course of infection. An example of such analysis is presented in Fig. 3. The rate of synthesis of proteins present in the ribosomal fraction was estimated in mock-infected cells and in cells infected for 6 h corresponding to the early stage of infection (Fig. 3C and D). In parallel, the rate of a sub-set of total cellular proteins was also estimated (Fig. 3A and B).

As expected for total cellular proteins, the synthesis rate of host proteins decreased dramatically in 6-h infected cells (compare Fig. 3A and B). The global synthesis rate of this set of proteins was decreased to ~40% of that of mock-infected cells. This reflected the typical HSV-1-induced shut-off of the majority of host protein syntheses. Conversely, the synthesis of all the basic ribosomal proteins separated in system II remained very efficient (compare Fig. 3C and D). Indeed the synthesis of ribosomal proteins was sustained until 9 h post-infection and finally decreased for most of them at 15 h post-infection [6,7].

4. Conclusions

Ribosomal protein maps have not yet been established using IEF. This is mainly due to the very basic nature of ribosomal proteins which renders them very difficult to separate using this method. However, several two-dimensional electrophoretic systems have been developed over the last two decades which allow a reproducible separation of all ribosomal proteins from procaryotic as well as from eucaryotic cells. Among them, four different but related systems were developed by Madjar and his colleagues which allowed several 2-D ribosomal protein maps to be built. Over the past few years the modifications of ribosomes and protein synthesis after infection by HSV-1 have been analyzed. This has been achieved by comparison of the ribosomal protein maps built from non-infected cells with those built from infected cells. These comparisons demonstrated that infection by HSV-1 induces important modifications of ribosomes: (i) non-reversible phosphorylation of ribosomal protein S6; (ii) unusual phosphorylation of several proteins of small and large subunits; and (iii) association of viral and cellular proteins to the ribosomal fraction. The role that these modifications may play in the regulation of viral and cellular gene expression remains to be determined. However, these modifications are correlated with changes in the cellular protein synthesis occurring during the course of infection. This observation led us to hypothesize that ribosomes themselves participate in the translational regulation of viral and cellular gene expression. The finding that several viral and cellular proteins with mRNA-binding activities associate with ribosomes after infection and that the synthesis of these modified ribosomes persists even late in infection, strongly supports this hypothesis. Indeed, one might postulate that these modified, and newly synthesized ribosomes allow the specific translation of some class of viral and/or cellular mRNAs whose translation is determinant for the outcome of infection.

5. Nomenclature

2-D	two-dimensional
2-DE	two-dimensional electrophoresis

DTE	dithioerythritol
HSV-1	herpes simplex virus type 1
IEF	isoelectric focusing
IPG	immobilized pH gradient
PABP	poly(A)-binding protein
PAGE	polyacrylamide gel electrophoresis
PFU	plaque forming unit
PVDF	polyvinylidene difluoride
SDS	sodium dodecyl sulfate
VP	viral protein

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References

[1] A. Greco, A.M. Laurent, J.-J. Madjar, Mol. Gen. Genet. 256 (1997) 320.
[2] D. Simonin, J.-J. Diaz, K. Kindbeiter, L. Denoroy, J.-J. Madjar, Electrophoresis 16 (1995) 854.
[3] D. Simonin, J.-J. Diaz, T. Massé, J.-J. Madjar, J. Gen. Virol. 78 (1997) 435.
[4] J.-J. Diaz, D. Simonin, T. Massé, P. Deviller, K. Kindbeiter, L. Denoroy, J.-J. Madjar, J. Gen. Virol. 74 (1993) 397.
[5] T. Massé, D. Garcin, B. Jacquemont, J.-J. Madjar, Mol. Gen. Genet. 220 (1990) 1.
[6] A. Greco, N. Bausch, Y. Couté, J.-J. Diaz, Electrophoresis 21 (2000) 2522.
[7] A. Greco, W. Bienvenut, J.C. Sanchez, K. Kindbeiter, D. Hochstrasser, J.-J. Madjar, J.-J. Diaz, Proteomics 1 (2001) 545.
[8] B. Roizman, in: B. Roizman, R.J. Whitley, C. Lopez (Eds.), The Human Herpesviruses, Raven Press, New York, 1993, p. 1.
[9] R.J. Whitley, J.W. Gnann Jr., in: B. Roizman, R.J. Whitley, C. Lopez (Eds.), The Human Herpesviruses, Raven Press, New York, 1993, p. 69.
[10] B. Roizman, A.E. Sears, in: B. Roizman, R.J. Whitley, C. Lopez (Eds.), The Human Herpesviruses, Raven Press, New York, 1993, p. 11.
[11] M.L. Fenwick, Virology 77 (1977) 860.
[12] M.L. Fenwick, M.J. Walker, J. Gen. Virol. 41 (1978) 37.
[13] E. Harris-Hamilton, S.L. Bachenheimer, J. Virol. 53 (1985) 144.
[14] R.W. Honess, B. Roizman, Proc. Natl. Acad. Sci. USA 72 (1975) 1276.
[15] S. Silverstein, D.L. Engelhardt, Virology 95 (1979) 334.
[16] M.L. Fenwick, M.J. Walker, J. Gen. Virol. 45 (1979) 397.
[17] I.M. Kennedy, W.S. Stevely, D.P. Leader, J. Virol. 39 (1981) 359.
[18] T. Massé, D. Garcin, B. Jacquemont, J.-J. Madjar, Eur. J. Biochem. 194 (1990) 287.
[19] H. Fraenkel-Conrat, Virology 4 (1957) 1.
[20] J.R. Waller, J.I. Harris, Proc. Natl. Acad. Sci. USA 47 (1961) 18.
[21] J.-J. Madjar, M. Arpin, M. Buisson, J.P. Reboud, Mol. Gen. Genet. 171 (1979) 121.
[22] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[23] U.K. Laemmli, Nature 227 (1970) 680.
[24] B.R. Oakley, D.R. Kirsch, N.R. Morris, Anal. Biochem. 105 (1980) 361.
[25] J.-J. Madjar, in: J.E. Celis (Ed.), Cell Biology: A Laboratory Handbook, Academic Press, London, 1994, p. 657.
[26] A. Greco, J.-J. Madjar, in: J.E. Celis (Ed.), Cell Biology: A Laboratory Handbook, Academic Press, London, 1998, p. 135.
[27] J.-J. Madjar, R.R. Traut, Mol. Gen. Genet. 179 (1980) 89.
[28] I.G. Wool, Y.-L. Chan, A. Glück, in: J.W.B. Hershey, M.B. Mathews, N. Sonenberg (Eds.), Translational Control, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1996, p. 685.
[29] A. Gorg, C. Obermaier, G. Boguth, A. Csordas, J.J. Diaz, J.J. Madjar, Electrophoresis 18 (1997) 328.
[30] E. Kaltschmidt, H.G. Wittmann, Anal. Biochem. 36 (1970) 401.
[31] E.H. McConkey, H. Bielka, J. Gordon, S.M. Lastick, A. Lin, K. Ogata, J.P. Reboud, R.R. Traugh, J.R. Warner, H. Welfe, I. Wool, Mol. Gen. Genet. 169 (1979) 1.
[32] J.-J. Madjar, S. Michel, A.J. Cozzone, J.P. Reboud, Anal. Biochem. 92 (1979) 174.
[33] M.J. Dunn, in: A. Chrambach, M.J. Dunn, B.J. Radola (Eds.), Advances in Electrophoresis, VCH, Weinheim, 1987, p. 1.
[34] B. Buendia, A. Person-Fernandez, G. Beaud, J.-J. Madjar, Eur. J. Biochem. 162 (1987) 95.
[35] J.-J. Diaz, O. Gandrillon, D. Hentzen, D. Leguellec, J. Samarut, J.-J. Madjar, Oncol. Res. 1 (1989) 163.
[36] J.-J. Madjar, K. Nielsen-Smith, M. Frahm, D.J. Roufa, Proc. Natl. Acad. Sci. USA 79 (1982) 1003.
[37] J.-J. Madjar, A. Fournier, Eur. J. Biochem. 163 (1987) 577.
[38] Y.-L. Chan, J.-J. Diaz, L. Denoroy, J.-J. Madjar, I.G. Wool, Biochem. Biophys. Res. Commun. 225 (1996) 952.
[39] M. Scott, D.L. Crimmins, D.W. McCourt, J.J. Tarrand, M.C. Eyerman, M.H. Nahm, Biochem. Biophys. Res. Commun. 155 (1988) 1353.
[40] H. Schägger, G. Von Jagow, Anal. Biochem. 166 (1987) 368.
[41] E. Hildebrandt, V.A. Fried, Anal. Biochem. 177 (1989) 407.
[42] G. Elliott, P. O'Hare, J. Virol. 73 (1999) 4110.
[43] M. Miranda-Saksena, P. Armati, R.A. Boadle, D.J. Holland, A.L. Cunningham, J. Virol. 74 (2000) 1827.
[44] U.A. Bommer, F. Noll, G. Lutsch, H. Bielka, FEBS Lett. 111 (1980) 171.

[45] F. Noll, U.A. Bommer, G. Lutsch, H. Theise, H. Bielka, FEBS Lett. 87 (1978) 129.
[46] H. Mao, S.A. White, J.R. Williamson, Nat. Struct. Biol. 6 (1999) 1139.
[47] J. Vilardell, S.J. Yu, J.R. Warner, Mol. Cell 5 (2000) 761.
[48] J. Vilardell, P. Chartrand, R.H. Singer, J.R. Warner, RNA 6 (2000) 1773.
[49] O. Meyuhas, Eur. J. Biochem. 267 (2000) 6321.
[50] I.G. Wool, Trends Biochem. Sci. 21 (1996) 164.
[51] H. Naora, Immunol. Cell Biol. 77 (1999) 197.
[52] R.J. Roller, B. Roizman, J. Virol. 66 (1992) 3624.
[53] R.J. Roller, L.L. Monk, D. Stuart, B. Roizman, J. Virol. 70 (1996) 2842.
[54] K.A. Cassady, M. Gross, B. Roizman, J. Virol. 72 (1998) 8620.
[55] K.A. Cassady, M. Gross, B. Roizman, J. Virol. 72 (1998) 7005.
[56] J.-J. Diaz, M. Duc Dodon, N. Schaerer-Uthurralt, D. Simonin, K. Kindbeiter, L. Gazzolo, J.-J. Madjar, Nature 379 (1996) 273.
[57] D.R. Gallie, Gene 216 (1998) 1.
[58] E. Wahle, A. Lustig, P. Jeno, P. Maurer, J. Biol. Chem. 268 (1993) 2937.
[59] A. Nemeth, S. Krause, D. Blank, A. Jenny, P. Jeno, A. Lustig, E. Wahle, Nucleic Acids Res. 23 (1995) 4034.
[60] C. Grosset, C.Y. Chen, N. Xu, N. Sonenberg, H. Jacquemin-Sablon, A.B. Shyu, Cell 103 (2000) 29.
[61] D.R. Gallie, Plant Mol. Biol. 32 (1996) 145.
[62] S.E. Wells, P.E. Hillner, R.D. Vale, A.B. Sachs, Mol. Cell 2 (1998) 135.
[63] H. Imataka, A. Gradi, N. Sonenberg, EMBO J. 17 (1998) 7480.
[64] Y. Ishigaki, X. Li, G. Serin, L.E. Maquat, Cell 106 (2001) 607.