Occurrence of Novel Small RNAs with Concomitant Inhibition of Host Cellular U Small Nuclear RNA Synthesis in Vero Cells Infected with Herpes Simplex Virus Type 1

By MICHAEL BACHMANN, DIETRICH FALKE, JÜRGEN PREUHS, HEINZ C. SCHRÖDER, KARIN PFEIFER and WERNER E. G. MÜLLER

1 Institut für Physiologische Chemie, Abteilung Angewandte Molekularbiologie, Universität, Duesbergweg, 6500 Mainz and 2 Institut für Medizinische Mikrobiologie, Abteilung Experimentelle Virologie, Universität, Obere Zahlbacher Strasse 67, 6500 Mainz, F.R.G.

(Accepted 26 August 1986)

SUMMARY

In eukaryotic cells, small nuclear and small cytoplasmic RNAs (sn- or scRNAs) are associated with distinct proteins, forming ribonucleoproteins (snRNPs or scRNPs). In the present study we analysed the protein composition as well as the small RNA pattern in non-infected and herpes simplex virus type 1 (HSV)-infected Vero cells. We found that concomitantly with the shut-off of host cell mRNA synthesis, synthesis of U-snRNAs was stopped. Due to their stability, however, U-snRNAs were still present in cells 36 h after HSV infection. Besides these RNAs, two novel small RNAs which we termed HVR1 and HVR2 were detected in infected cells. On the basis of their relative mobilities in urea gels, the apparent chain lengths of these newly synthesized RNAs were determined to be 255 and 154 nucleotides respectively. The small RNA-binding proteins Sm, RNP, Ro and La were found to increase up to 15-fold after HSV infection. The data presented suggest that new, virus-coded small RNPs are synthesized which might play a role in the maturation and regulation of HSV-coded RNA transcripts.

INTRODUCTION

Based on the appearance of distinct, virus-coded proteins, the infectious cycle of herpes simplex virus type 1 (HSV-1) is divided into three stages: immediate early, early and late (Jones & Roizman, 1979). In contrast to HSV-coded mRNAs, host cell mRNAs are rapidly degraded after infection and their de novo synthesis is suppressed due to an inhibition of RNA polymerase II transcribing cellular genes (Nishioka & Silverstein, 1977; Inglis, 1982). Some exceptions are known in which an increase in synthesis of distinct host cell proteins occurs after infection, e.g. histone H3 (Mayman & Nishioka, 1985). This is attributed to events at the post-transcriptional level, resulting in stabilization of histone H3 mRNA (Mayman & Nishioka, 1985). Cellular mRNAs are deadenylated and thereby destabilized (Silverstein et al., 1973; Orellana & Kieff, 1977; Müller et al., 1978a).

During infection by DNA viruses additional small RNAs are formed, for instance during adenovirus infection the VA-RNAs or during Epstein–Barr virus infection the EBER-RNAs (Rosa et al., 1981; Lerner et al., 1981a). These low molecular weight RNAs are transcribed by RNA polymerase III and belong to the group of La small nuclear (sn) RNAs (Rosa et al., 1981; Francoer & Mathews, 1982). The La protein is known to be one of the antigenic targets that are recognized by IgGs from patients with autoimmune diseases, for example systemic lupus erythematosus (Wasicek & Reichlin, 1982). Besides La the most familiar antigen–antibody systems are the Ro, Sm or RNP systems or combinations of them (Lerner et al., 1982; Lerner & Steitz, 1981). All of these proteins form complexes (ribonucleoproteins, RNP) with snRNAs or small cytoplasmic RNAs (scRNAs) resulting in the assembly of snRNPs or scRNPs (Lerner &
Some of the snRNAs are thought to be involved in splicing of nuclear premessengers (Busch et al., 1982); e.g. U1- and U2-snRNA.

In the present study it is shown for the first time that some hitherto unknown small RNAs which we have termed HVR1 and HVR2 are synthesized in HSV-infected cells. Moreover it was found that the transcription of U-snRNAs by RNA polymerase II is inhibited, while the synthesis of small RNA-binding protein increases. Despite this, U-snRNAs are not degraded during the HSV infectious cycle.

**METHODS**

**Materials.** $[^{32}P]$Phosphorus (4-7 Ci/mmol) and $[^{32}P]dTTP (3000$ Ci/mmol) were from Amersham, Protein A-Sepharose from Pharmacia, 4-chloro-1-naphthol and peroxidase-conjugated antibodies [anti-human γ-chain specific (no. A 8775) and anti-mouse (no. A 5278)] from Sigma, nitrocellulose sheets (BA85; 0.45 μm; no. 40 1180) from Schleicher & Schuell, nitrocellulose filter discs (HAWP; 0.45 μm) from Millipore and Zeta-Probe blotting membranes from Bio-Rad.

**Cells and viruses.** HSV-1 strain Lennette was grown in Vero cells as described by Müller et al. (1978b). The cell monolayers (4 × 10^7 cells/flask) were infected at 5 p.f.u./cell. Under these conditions, viral DNA synthesis starts 3 h after infection and reaches a maximum after 7 h (Müller & Zahn, 1979).

**Extracts.** For analysis of the proteins associated with RNPs, 4 × 10^7 cells were harvested by centrifugation (10000 g; 10 min; 4°C) 2, 4 or 8 h after virus infection. After washing of each cell sample (three times) with 1 ml of 0.9% NaCl, they were resuspended in 1 ml of lysis buffer [10 mM Tris–HCl pH 7.4, 350 mM NaCl, 15 mM MgCl2, 1% (v/v) Nonidet P40, 0.2% (w/v) SDS including the protease inhibitors leupeptin, pepstatin and chymostatin (5 μg/ml each) and 0.5 mM-phenylmethylsulphonyl fluoride]. Cells were broken and homogenized using a Dounce Potter homogenizer (10 strokes) with an S pestle. Extracts were collected by centrifugation (10000 g; 15 min; 4°C).

For analysis of RNA in RNPs, uninfected or HSV-infected cells were grown in Hanks’ medium (Lonsdale, 1979). Incubation was performed in flasks (7 × 10^6 cells). The cells were harvested 4 h or 36 h after infection. For RNA labelling, 1 mCi $[^{32}P]$ was added to the control and virus-infected cells immediately after infection. The cells were resuspended in 500 μl of RNA extraction buffer [100 mM-sodium acetate pH 5.5, 7 M-urea, 2% (w/v) SDS] and homogenized as described above. Two-hundred and fifty μl of phenol (equilibrated with RNA extraction buffer) was added, followed by 10 strokes in the Dounce Potter homogenizer. Then 250 μl of chloroform and 10 μl of isooamyl alcohol were added, and the RNA was extracted at 55°C as described by Bachmann et al. (1984).

**RNA analysis.** RNA was extracted from cultures grown in the presence of $[^{32}P]$, as described above. Prior to electrophoresis, the RNA was collected by centrifugation (10000 g; 5 min; 4°C), re-dissolved in 350 mM-NaCl (250 μl) and precipitated again with ethanol (875 μl final volume). After repeating this procedure, the total amount of nucleic acids from 7 × 10^6 cells was dissolved in 200 μl of RNA electrophoresis buffer (Bachmann et al., 1984), supplemented with 8 M-urea and 15% (v/v) glycerol. Aliquots (5 μl or 50 μl) were analysed by 10% polyacrylamide/7 M-urea gel electrophoresis (Kinlaw et al., 1982). Prior to electrophoresis samples were heated for 10 min at 95°C.

After electrophoresis, gels containing labelled RNA were dried and exposed to Kodak X-Omat film for 4 h or 14 days. Unlabelled RNAs were visualized by silver staining (McNeilage & Wittingham, 1984); the sensitivity of this method was determined to be as low as 0.2 ng of RNA.

The chain length of the RNAs was determined on the basis of their relative mobilities in the gels according to Bachmann et al. (1984).

**Northern blot analysis.** HSV DNA was isolated from 3 × 10^7 Vero cells (12 h post-infection), following the procedure of Farber (1976). The HSV DNA was not contaminated with cellular DNA, as analysed by CsCl buoyant density isopycnic centrifugation (Müller et al., 1977). The viral DNA was nick-translated with $[^{32}P]dTTP according to Rigby et al. (1977). Chromatography on Sephadex G-50 (Maniatis et al., 1982) yielded a probe with a specific radioactivity of 3.9 × 10^7 c.p.m./μg DNA. Unlabelled RNA samples were treated at 95°C for 10 min and electrophoresed on 10% polyacrylamide/7 M-urea gels as described above. After electrophoresis, the separated RNAs were transferred from the gel to Zeta-Probe blotting membrane (Church & Gilbert, 1984) and then hybridized with the $[^{32}P]$-labelled HSV DNA according to Maniatis et al. (1982) and Messer et al. (1986). The filters were washed under stringent conditions (Messer et al., 1986), dried and subsequently subjected to autoradiography with Kodak XAR-5 X-ray film and DuPont Lightning-Plus intensifying screens at −80°C.

**Sera and monoclonal antibodies.** Reference antisera were obtained from the Centers for Disease Control (CDC), Atlanta, Ga., U.S.A. A control serum was obtained from a healthy rabbit; the resulting IgG preparation gave no precipitin line either with human spleen or rabbit thymus extract (Sharp, 1981). Sera from patients with autoimmune disorders were screened for anti-Sm or anti-RNP antibodies. They were standardized by counterimmunoelectrophoresis (CIE) using the CDC reference sera (Bachmann et al., 1984). The following sera
Novel small RNAs in HSV-infected cells

were chosen: Ko-IgG (anti-Sm) and Su-IgG (anti-RNP). IgG fractions were prepared by ammonium sulphate precipitation, and chromatography on DE-52 and Protein A-Sepharose (Hjelm et al., 1972). Another IgG fraction, termed Fi-IgG, was found by CIE to be directed against the Sm, RNP, Ro and La antigens. Monoclonal antibodies against purified homogeneous La and Ro protein were prepared as described (Bachmann et al. 1986a,b). They were shown to be directed against the 50000 mol. wt. La protein or the 95000 (60000) mol. wt. Ro protein (Bachmann et al., 1986b).

Immunoblotting. Prior to electrophoresis 200 μl protein extracts from 4 x 10⁷ uninfected or HSV-infected cells were concentrated (Bachmann et al., 1984). The pelleted proteins were redissolved in 10 μl sample buffer, electrophoresed in the presence of SDS in 10% polyacrylamide gels (Laemmli, 1970). Then the proteins were transferred to nitrocellulose sheets (Towbin et al., 1979). After blocking with bovine serum albumin, the nitrocellulose was incubated with a rabbit serum to reduce non-specific staining. Antigens were identified using Fi-IgG. Immunocomplexes were visualized with anti-human IgG (peroxidase-conjugated) using 4-chloro-1-naphthol/hydrogen peroxide as substrate (Gramzow et al., 1986).

La antigen. Homogeneous La protein was purified as described by Bachmann et al. (1986a,b). Briefly, L5178y cell extract was treated with ribonuclease A and fractionated by ammonium sulphate precipitation, Sephadex G-150 gel filtration and subsequent immunoaffinity chromatographic steps, using monoclonal anti-La and anti-Ro columns. The mol. wt. of purified La was determined to be 50000.

ELISA test. Five μl of cell extract was soaked through HAWP filters. After blocking with blocking solution (1% bovine serum albumin in 10 mM-Tris-HCl pH 7.4; 150 mM-NaCl), filters were incubated (1 h) with control rabbit IgG to reduce non-specific binding. Then the filters were incubated (1 h; 20 °C) in the presence of monoclonal anti-La and anti-Ro (0.2 μg/filter), or monospecific anti-Sm or anti-RNP (100 μg/filter) antibodies. The non-bound IgGs were removed by washing three times with 5 ml of blocking solution supplemented with 0.5% Tween 20, followed by three washing steps with blocking solution only. After treatment with anti-mouse or anti-human IgG (peroxidase-conjugated; diluted 1:500 with blocking solution), the filters were incubated with 200 μl of substrate solution (2.25 mM-o-phenylenediamine, 4 mM-hydrogen peroxide in 50 mM-phosphate/citrate buffer pH 5.0) for 5 min at 20 °C. Samples (100 μl) were withdrawn and the peroxidase reaction was stopped by addition of 100 μl of 0.2 m-sulphuric acid. Absorbance was determined at 492 nm. The calibration curve was found to be linear up to 1 μg of antigen. For the purpose of comparison, the absorbance of the assay sample from non-infected cells was set at 1 and the absorbance of the corresponding sample from HSV-infected cells was read against this value (i.e. in arbitrary units).

RESULTS

The occurrence of small RNPs in uninfected and HSV-1-infected cells was investigated with respect to their protein and RNA composition.

RNP-associated proteins

Four x 10⁷ uninfected or HSV-1-infected Vero cells were used to prepare extracts and proteins were separated by polyacrylamide gel electrophoresis as described in Methods. By immunoblotting using a purified IgG fraction from a patient with an autoimmune disorder (Fi-IgG) which contained antibodies against Sm, RNP, Ro and La, the corresponding antigens were visualized in virus-infected cells. As shown in Fig. 1, the amount of Sm and RNP increased considerably in extracts of HSV-infected cells (2 h, Fig. 1 d; 4 h, Fig. 1 c; 8 h, Fig. 1 b) and, more strikingly, the La antigen appeared as a distinct band on the immunoblot of the fractions from infected cells. In lane (a) of Fig. 1, homogeneous La antigen was analysed and was used as an internal standard to identify the antigens. The Ro antigen remained undetected under the conditions used. Lane (e) of Fig. 1 shows the reaction of the antibodies with an extract of uninfected cells. The protein concentration of this sample was twice that in lane (d). In spite of this, only the La and Sm antigens were detected under these conditions, indicating again the increase of the antigens after virus infection.

This increase in antigen concentration of HSV-infected cells was additionally quantified by ELISA (Table 1). For this determination we used monoclonal anti-La and anti-Ro antibodies or monospecific anti-Sm and anti-RNP antibodies. The amounts of all antigens were found to increase after infection. The increase was highest for La (15-fold), moderate for Sm (fivefold) and RNP (fourfold) and only small for Ro. The time course study shows that the induction of antigen synthesis coincides with the onset of HSV DNA synthesis (Müller & Zahn, 1979; Müller et al., 1983).
Fig. 1. Western blot analysis of RNP, Sm, Ro and La antigens in virus-infected cells. Total protein extracts from HSV-infected cells, 2 h (d), 4 h (c), or 8 h (b) post-infection were analysed by immunoblotting as described in Methods using Fi-IgG. (a) Homogeneous La antigen. Markings are taken from Bachmann et al. (1985). An extract of uninfected cells was run in lane (e).

Table 1. Changes in the amounts of La, Ro, Sm and RNP antigens in Vero cells as a function of time after HSV infection*

<table>
<thead>
<tr>
<th>Antibody</th>
<th>0 h</th>
<th>2 h</th>
<th>4 h</th>
<th>8 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-La</td>
<td>1</td>
<td>7.3</td>
<td>15.3</td>
<td>15.1</td>
</tr>
<tr>
<td>Anti-Ro</td>
<td>1</td>
<td>1.1</td>
<td>1.2</td>
<td>1.1</td>
</tr>
<tr>
<td>Anti-Sm</td>
<td>1</td>
<td>1.9</td>
<td>4.9</td>
<td>4.5</td>
</tr>
<tr>
<td>Anti-RNP</td>
<td>1</td>
<td>1.7</td>
<td>4.3</td>
<td>3.9</td>
</tr>
</tbody>
</table>

* Determinations were performed by an ELISA technique, described in Methods. The means of five parallel experiments are given; s.d. was less than 5%.

Small RNA pattern in HSV-infected cells

RNA extracted from uninfected or HSV-infected Vero cells was separated by gel electrophoresis in the presence of 8 M-urea and visualized by two techniques, namely a silver staining procedure and autoradiography. For the latter approach, the RNA was labelled by growing the cells in the presence of $^{32}$P. The application of these two methods, performed in parallel, enabled discrimination between stable (detection by silver staining) and newly synthesized small RNA (visualization by autoradiography).

Using the silver stain procedure (Fig. 2), all U-snRNAs (U1, U2, U4, U5 and U6) as well as 4S, 4.5S, 5S and 5.8S RNAs were shown to be present in the RNA preparations from HSV-infected (lanes a and b) and uninfected Vero cells (lanes c and d). However, in the RNA preparation from HSV-infected cells two new small RNAs appeared which had relative mobilities of 0.29 and 0.56.

The most likely explanation of their existence, i.e. that these two RNAs were newly synthesized after HSV infection, was experimentally proven by labelling the RNAs in intact cells with $^{32}$P. The analysis of the $^{32}$P-labelled RNA by gel chromatography and subsequent autoradiography again revealed two new bands in the RNA preparation from HSV-infected cells (Fig. 3). Their relative mobilities were identical with those determined for the RNAs in the silver-stained gels (Fig. 2). These RNAs were termed HVR1 and HVR2. They were separated into two species on the silver-stained gel (Fig. 2). The autoradiograph (Fig. 3) shows that HVR1 was highly labelled, whereas HVR2 appeared only as a faint band. Moreover, the labelling experiment revealed that in HSV-infected cells the de novo synthesis of U-snRNAs, especially of U1- and U2-snRNAs, is drastically reduced or abolished. This conclusion is drawn from the autoradiogram (Fig. 3), which shows the bands for the mentioned U-snRNAs only in uninfected...
Novel small RNAs in HSV-infected cells

Fig. 2. Polyacrylamide gel electrophoresis of unlabelled RNA from uninfected or HSV-infected Vero cells. The gel was silver-stained as described in Methods. RNA was from infected cells, (a) 50 μl sample and (b) 5 μl sample or from uninfected cells, (c) 5 μl sample and (d) 50 μl sample. The positions of the tRNAs, 5S and 5.8S RNAs as well as of U-snRNAs were determined in a previous study (Bachmann et al., 1985). The positions of HVR1 and HVR2 are indicated.

Fig. 3. Polyacrylamide gel electrophoresis (in the presence of urea) of 32P-labelled RNA from uninfected or HSV-infected Vero cells. The RNA was extracted 4 h after infection. Dried gels were autoradiographed for 4 h (a) or 14 days (b). RNA from (lanes 1) control cells, 50 μl sample; (lanes 2) virus-infected cells, 50 μl sample. The positions of HVR1 and HVR2 are indicated by arrows. Further details are given in the legend to Fig. 2.

(lanes 1) but not in infected cells (lanes 2). These U-snRNAs did not undergo degradation in infected cells, because they were visible in silver-stained gels (Fig. 2, lane a).

Based on their relative mobilities and using 5S and 5.8S rRNAs as standards, the apparent chain lengths of the newly synthesized RNAs were calculated for HVR1 to be 255 nucleotides and for HVR2 154 nucleotides.
In a second approach, RNA samples from uninfected or HSV-infected Vero cells (4 h post-infection) were isolated and electrophoresed on polyacrylamide gels. Blot-transferred RNA was hybridized with $^{32}$P-labelled HSV DNA. The resulting autoradiogram is shown in Fig. 4. The sizes of the two RNA bands were estimated by the use of appropriate RNA size standards. The values for the slowly migrating RNA as well as for the rapidly migrating RNA correspond exactly to those calculated as described above for the HVR1 and HVR2 RNA species (Fig. 3). HVR1 is the predominant RNA species (Fig. 4, lane c). To identify HVR2 and HVR1 an RNA sample at a 10-fold higher concentration was run in Fig. 4, lane (a). RNA samples from control cells did not contain HVR1 or HVR2 (Fig. 4, lane b).

**DISCUSSION**

Little is known about the fate of RNA polymerase II transcripts that do not code for proteins during HSV infection. In particular, the metabolism of U-snRNAs in HSV-infected cells has not yet been studied. Some of the U-snRNA-binding proteins are the antigenic targets of IgGs present in sera of patients with autoimmune diseases (Wasicek & Reichlin, 1982). While the U-snRNAs which are associated with the Sm or RNP antigen are transcribed by RNA polymerase II, those small RNAs which form a particle with the La antigen are predominantly RNA polymerase III transcripts (Busch et al., 1982; Rinke & Steitz, 1982). In some cases the cellular La-RNAs are also RNA polymerase II transcripts, e.g. U1-snRNA precursor (Rinke & Steitz, 1982); they are predominantly localized in the nucleus. The Ro-RNAs, which are presumably a subclass of the La-RNAs, are found primarily in the cytoplasm (Hendrick et al., 1981).

In contrast to the adenovirus or Epstein-Barr virus systems (Rosa et al., 1981; Lerner et al., 1981a) no data are available about small RNAs present during HSV infection. In the first part of this study we quantified the antigenic proteins La, Ro, Sm and RNP. All these antigens increased after HSV infection. The increase of La especially (15-fold) was considerable. The amount of Ro increased only slightly (1.5-fold). The increase in proteins associated with RNPs can be attributed to changes at the post-transcriptional level, as has been established for the H3 histone protein (Mayman & Nishioka, 1985).

In a second set of experiments we analysed the pattern of small RNAs in HSV-infected cells. It is known that U-snRNAs are stable in uninfected cells for more than 48 h (Busch et al., 1982;
Novel small RNAs in HSV-infected cells

Brunel et al., 1985). Our studies revealed that total RNA extracts from HSV-infected cells still contained U-snRNA at 36 h after infection. This finding came from analyses of urea gels, stained with silver. No conclusion can be drawn by this approach about the rate of synthesis of the respective small RNA. Therefore we labelled the small RNAs with $^{32}$P and found that the de novo synthesis of U-snRNAs is blocked in infected cells. On the other hand, two novel small RNAs, termed HVR1 and HVR2, were synthesized immediately after infection. According to their mobility in the urea gels, we estimated for HVR1 a nucleotide chain length of 255 nucleotides and for HVR2 a length of 154 nucleotides. The mobility of HVR1 is in the range of the adenovirus VA-RNA (Lerner et al., 1981b). In view of earlier findings (Lerner et al., 1981b) the exact chain lengths of the HVRs can be given only after their sequencing. We assume that both HVR1 and HVR2 are virus-coded, because they are absent in control cells and are only synthesized during the infection cycle as indicated by their radiolabelling. This view is supported by Northern blot analyses using $^{32}$P-labelled HSV DNA which revealed that the RNA species HVR1 and HVR2 are present only in RNA samples from HSV-infected cells and are absent from RNA samples from uninfected cells. Further analyses are in progress to clarify the origin of HVR1 and HVR2 and their possible relationships to VA- or EBER-RNAs.

This investigation was supported by a grant from the Deutsche Forschungsgemeinschaft (Mu 348/7-5).

REFERENCES


(Received 13 May 1986)