Variability in inhibition of host RNA synthesis by entero- and cardioviruses

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Both entero- and cardioviruses have been shown to suppress host mRNA synthesis. Enteroviruses are also known to inhibit the activity of rRNA genes, whereas this ability of cardioviruses is under debate. This study reported that mengovirus (a cardiovirus) suppressed rRNA synthesis but less efficiently than poliovirus (an enterovirus). In contrast to poliovirus infection, the incorporation of BrUTP, fluorouridine and [14C]uridine in rRNA precursors was observed even during the late stages of mengovirus infection, although at a significantly reduced level. The cleavage of TATA-binding protein, considered to be one of the central events in poliovirus-induced transcription shutoff, was not detected in mengovirus-infected cells, indicating a difference in the mechanisms of host RNA synthesis inhibition caused by these viruses. The results also showed that functional leader protein is redundant for the suppression of host RNA synthesis by cardiovirus.

Protease 3Cpro of poliovirus (genus Enterovirus) has been shown to inhibit the synthesis of all three cellular RNA polymerases (pol I–III) as a result of the degradation of several transcription factors (Banerjee et al., 2005; Clark et al., 1993; Fradkin et al., 1987; Weidman et al., 2001; Yalamanchili et al., 1997b, c). Encephalomyocarditis virus (EMCV), from the distantly related genus Cardiovirus, has also been demonstrated to inhibit mRNA production (Apriletti & Penhoet, 1978), whereas its ability to suppress rRNA synthesis is under debate. A recent observation suggested that EMCV has no effect on rRNA production (Aminev et al., 2003), whereas an earlier study reported that the synthesis of rRNA in infected cells was inhibited (Plagemann, 1968). The goal of the current study was to clarify this issue in a comparative study with poliovirus.

To reveal the patterns of host transcription after picornavirus infection, we stained cells with 5-bromouridine 5′-triphosphate (BrUTP) in situ. To this end, HeLa-B cells (Tolskaya et al., 1995) grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum were split 1 day before the experiment and seeded on glass coverslips in a 12-well plate so that they formed a subconfluent monolayer the next day. The cells were infected with poliovirus 1 strain Mahoney and the mengovirus strain of EMCV at an m.o.i. of ~100 p.f.u. per cell, as described previously (Bardina et al., 2009). At the indicated times, the cells were permeabilized with digitonin (40 μg ml−1) in glycerol buffer [25% (w/v) glycerol, 5 mM MgCl2, 0.5 mM EGTA, 0.5 mM PMSF, 20 mM Tris/HCl (pH 7.4)] and exposed to transcription buffer [25% (w/v) glycerol, 100 mM KCl, 5 mM MgCl2, 0.5 mM EGTA, 0.5 mM each ATP, CTP and GTP, 0.2 mM BrUTP (Sigma), 1 mM PMSF, 5 U RNAsin ml−1 (Promega), 50 mM Tris/HCl (pH 7.4)] for 30 min at 37 °C as described previously (Kobena et al., 2000). After incubation, the coverslips were fixed in cold 1:1 methanol:acetone mix at −20 °C for 4 min, dried and stained with anti-BrUTP monoclonal antibody (clone BU-33; Sigma) and Hoechst-33342 DNA dye as described previously (Bardina et al., 2009). The coverslips were...
placed onto Mowiol mounting solution (Calbiochem) and examined with a Leica DMLS or Zeiss Axiovert 200M microscope.

The control cells displayed a moderate granular staining in the nucleoplasm that corresponded to RNA pol II- and pol III-dependent synthesis, which could not be discriminated by this method. In the nucleolar area, the staining was concentrated in numerous bright foci revealing the locations of rRNA synthesis in fibrillar centres (Fig. 1a, left columns). After infection with both poliovirus and mengovirus, we observed a gradual increase in cytoplasmic fluorescence, most probably reflecting the activity of the viral RNA-dependent RNA polymerase. Both viruses similarly caused a significant reduction in incorporation of the label into the nucleoplasm, suggesting the shutoff of pol II- and probably pol III-driven RNA synthesis (Fig. 1a, b). The viruses had different effects on the pattern of nucleolar labelling. The nucleolar fluorescence in poliovirus-infected samples became undetectable in the majority of cells as early as 4 h p.i. (Fig. 1a, central columns), whilst in mengovirus-infected cells, the signal in the nucleoli was present until the latest stages of infection (6 h p.i.), although at a significantly lower level than in mock-infected cells (Fig. 1a, right columns). This residual fluorescence in cardiovirus-infected cells was concentrated in fewer fibrillar centres, which were increased in size compared with the control (Fig. 1c). A similar pattern of nucleolar alterations was described as being associated with decreased rRNA synthesis during cell differentiation (Zatsepina et al., 1988) or in response to other suppressive conditions (Jordan & McGovern, 1981). We confirmed that nucleolar RNA synthesis in mengovirus-infected cells is carried out by the host transcription machinery, as it could be inhibited by the addition of actinomycin D (2 μg ml⁻¹) at 30 min before permeabilization (Fig. 1d).

In order to rule out possible artefacts due to label incorporation into the nuclei of permeabilized cells, similar experiments were performed with live cells. Infected HeLa cells were labelled with fluorouridine (0.1 mM; Sigma) in fresh medium at 37 °C for 10 min, washed with PBS, permeabilized with 40 μg digitonin ml⁻¹ in the same buffer to reduce the background and processed for indirect immunofluorescence as described previously (Bardina et al., 2009). The pattern of nuclear changes in these experiments was identical to that observed in the BrUTP-labelled permeabilized cells (not shown). Similar results were obtained with BrUTP staining of mengovirus-infected BHK-21 cells (not shown), suggesting that the host RNA synthesis shutoff induced by cardiovirus is a general phenomenon manifested in cells of different origin.

To corroborate our results with a non-microscopy-based biochemical method, HeLa cells infected with polio- and mengovirus as described above were exposed to [³¹⁴C]uridine (2 μCi per 60 mm dish). After incubation for 1 h at 37 °C, the cells were detached with Versene, collected by centrifugation, resuspended in 0.5 ml TRI Reagent (Sigma) and processed for total RNA isolation according to the manufacturer’s protocol. The amount of material in the loaded samples was adjusted according to the absorbance at 260 nm, and the samples were resolved on a 1% denaturing glyoxal/agarose gel as described previously (Sambrook et al., 1989). The gels were stained with ethidium bromide (Fig. 1e) or dried and analysed using a phosphorimager (FujiFilm) (Fig. 1f). The two bands representing the 45S and 32S rRNA precursors surrounded by heterogeneous material, presumably corresponding to the mRNA transcripts (Fig. 1f, asterisk), were clearly visible in the control samples. After infection with both viruses, the amount of heterogeneous material was significantly decreased and the vast majority of the signal was concentrated in the actinomycin D-resistant bands corresponding to the viral RNA (Fig. 1e, f, arrowheads). In agreement with the results obtained with BrUTP staining, the residual 45S rRNA signal disappeared rapidly in poliovirus-infected cells, but was stabilized at a low but clearly detectable level until the late stages of mengovirus infection.

We thus showed that both viruses similarly inhibited host transcription, but that mengovirus-induced suppression of rRNA synthesis was significantly weaker compared with that observed in poliovirus-infected cells. This result was different from that reported previously (Aminev et al., 2003), presumably because of the higher resolution of our methods. We cannot exclude the possibility that this effect could depend on some unidentified conditions of cell cultivation; however, our results are supported by the experiments performed in two different cell lines. The functional role, if any, of the residual activity of rRNA genes for replication of mengovirus remains to be determined.

TATA-binding protein (TBP) is a highly conserved transcription factor that is necessary not only for the activity of TATA box-containing pol II promoters, but also for pol I- and III-driven transcription (Margottin et al., 1991; Zomerdijk et al., 1994). Poliovirus-induced degradation of TBP carried out by 3Cpro has been described previously in detail and is considered to be one of the central points of host transcription shutoff in infected cells (Das & Dasgupta, 1993; Yalamanchili et al., 1996). To elucidate the mechanisms underlying the observed dissimilarity in nuclear transcription inhibition in polio- and mengovirus-infected cells, we investigated the state of TBP in infected cells. To this end, cytoplasmic lysates from HeLa cells infected as in Fig. 1 were analysed by Western blotting with antibodies against TBP (Santa Cruz) and actin (Sigma). As shown in Fig. 2, the level of TBP from mengovirus-infected cells remained constant, even at 6 h p.i., in contrast to the drastic decrease in the level of TBP during the course of poliovirus infection. We concluded that the mechanism of host transcription shutoff induced by mengovirus is different from that in poliovirus-infected cells, at least in terms of involvement of this transcription factor.
It has been shown that virus-associated rearrangements in infrastructure and metabolism of the infected cell, although similar in appearance, could be driven by non-homologous viral 'security proteins' (Romanova et al., 2009). For example, the unrelated poliovirus protease 2Apro and cardiovirus leader protein have been shown to affect interferon signalling (Hato et al., 2007; Morrison & Racaniello, 2009), nuclear transport (Bardina et al., 2009; Belov et al., 2004; Castello et al., 2009; Belov et al., 2004; Castello et al., 2009;...
2Apro protease are involved in inhibition of apoptosis in studies have revealed that the leader protein and probably 1996) by utilizing obviously different mechanisms. Recent and host-cell translation (Krausslich et al., 1987; Zoll et al., 1996) by utilizing obviously different mechanisms. Recent studies have revealed that the leader protein and probably 2Apro protease are involved in inhibition of apoptosis in infected cells (Burgon et al., 2009; Romanova et al., 2009). These poliovirus-induced effects on cellular functions are considered to be based predominantly on proteolytic degradation of numerous host proteins (Belov et al., 2003; Etchison et al., 1982; Gustin & Sarnow, 2001), whereas cardioviruses are suggested to utilize the cellular phosphorylation-based mechanisms (Bardina et al., 2009; Gingras et al., 1996). Interestingly, poliovirus 2Apro has been shown to inhibit host transcription when expressed individually (Davies et al., 1991; Ventoso et al., 1998), but was unable to affect mRNA synthesis in vitro (Yalamanchili et al., 1997a). In order to reveal the possible involvement of mengovirus leader protein in the inhibition of host transcription, we tested two mutant mengoviruses with a disrupted zinc finger motif or with a mutation of the T47 phosphorylation site of the leader peptide. Both viruses were able to inhibit BrUTP incorporation in the nuclei of infected HeLa-3E cells (Fig. 3), in spite of the lack of at least some of the leader-dependent functions and a moderate replication deficiency (Hato et al., 2007; Lidsky et al., 2006; Romanova et al., 2009; Zoll et al., 1996, 2002). These data are in line with the recent evidence for accumulation of the newly synthesized poly(A)-containing RNA in the nuclei of cells ectopically expressing the leader protein from the related Thielier’s murine virus (Ricour et al., 2009). Thus, neither the intact leader itself nor leader-dependent permeabilization of the nuclear envelope (Bardina et al., 2009; Lidsky et al., 2006) is necessary for host transcription shutoff. Therefore, mengovirus-induced factor(s) seem to be able to affect the nuclear functions even when the nuclear envelope is intact.

The host transcription shutoff has been studied in poliovirus-infected cells and is considered to be executed by a conserved protease 3Cpro (Weidman et al., 2003). Whilst the exact cardiovirus protein(s) responsible for host-cell transcription shutoff are yet to be determined, our data highlight the differences in the mechanism of cardiovirus and enterovirus infection on the host transcription machinery. Cardioviruses displayed an inability to trigger the cleavage of TBP in infected cells. As the nucleocytoplasmic barrier becomes permeable for passive diffusion in cardiovirus-infected cells (Lidsky et al., 2006) and 3Cpro has been shown to localize directly in nuclei (Aminev et al., 2003), we suggest that resistance of TBP to proteolysis by cardiovirus 3Cpro, rather than spatial isolation, protects this protein from degradation.

Despite its similarity in all family members, 3Cpro has been shown to vary among different picornaviruses in proteolytic specificity (Blom et al., 1996; Parks et al., 1989; Williams et al., 2009) and in its effects on the host cell (Armer et al., 2008). Our data provide additional evidence for the functional diversity of 3Cpro and general variability in the mechanisms of picornavirus interactions with the host cell.

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