Degradation of cellular mRNA during influenza virus infection: its possible role in protein synthesis shutoff

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The kinetics of cellular mRNA decay in influenza virus-infected cells have been studied by means of blot hybridization using as probes cloned cDNAs of α- and β-actin, α- and β-tubulin and vimentin. Both cellular mRNAs isolated from the cytoplasmic fractions as well as total cell mRNAs showed a rapid decay, with up to 50% concentration reductions at infection times at which influenza virus M1 mRNA was still not detectable. In contrast, these cellular mRNAs were stable in uninfected cells. To ascertain the possible role of mRNA degradation in the cellular protein synthesis shutoff, the kinetics of protein synthesis in infected cells were examined by two-dimensional gel electrophoresis of extracts pulse-labelled at several times after viral infection. The synthesis of the cellular proteins was reduced, showing kinetics paralleling those of mRNA decay. It is proposed that influenza virus infection induces the destabilization of mRNAs and that this mRNA degradation is, at least in part, responsible for cellular protein synthesis shutoff.

Introduction

Many viruses induce, upon in vitro infection of susceptible cells, a general inhibition of cellular macromolecular synthesis referred to as the shutoff phenomenon. There are several levels at which different viruses interfere with cell gene expression to produce shutoff. Picornaviruses and vesicular stomatitis virus inhibit RNA polymerase II activity (Crawford et al., 1981; McGowan et al., 1982). Adenoviruses block the cellular mRNA nuclear-cytoplasmic transport (Babich et al., 1983). Herpesviruses and poxviruses induce the degradation of pre-existing cellular mRNAs (Nishioka & Silverstein, 1978; Rice & Roberts, 1983). Finally, viruses alter the cell translational machinery by several different means. Thus, inactivation of the p220 subunit of the eIF-4F initiation factor, under-phosphorylation of the cap-binding protein, phosphorylation of the eIF2 initiation factor induced by dsRNA, or increased permeability of the infected cell membrane to monovalent ions might be responsible for protein synthesis inhibition induced by poliovirus, adenovirus or encephalomyocarditis (EMC) virus, respectively (Alonso & Carrasco, 1981; Etchison et al., 1982, 1984; Schneider et al., 1985; Huang & Schneider, 1991; for a general review, see Kozak, 1986).

In the case of influenza viruses, previous reports give conflicting results regarding the mechanisms by which they inhibit cellular protein synthesis: Inglis (1982) reported the degradation of pre-existing cellular mRNAs as the relevant cause for protein synthesis decay during viral infection. On the other hand, Katze & Krug (1984) have proposed the establishment of a virus-specific translational system in influenza virus-infected cells, able to use viral mRNAs preferentially, in the absence of any substantial degradation of their cellular counterparts.

In the course of the study of viral mRNA stability, we used actin mRNA as an internal control for mRNA recovery and found unexpected and aberrant results. Hence, we decided to carry out a detailed study of the cellular mRNA stability during viral infection. Using a variety of cloned cell DNAs, in this report we show that cellular mRNAs decay rapidly in the course of influenza virus infection, with a parallel decay in the kinetics of synthesis of the corresponding proteins.

Methods

Biological materials. The MDCK cell line was obtained from the American Type Culture Collection at passage 53. The COS-1 cell line (Gluzman, 1981) was obtained from Y. Gluzman. Cell stocks were kept
frozen and the cells were used within the subsequent 15 passages. The influenza virus A/Victoria/3/75 strain was plaque-purified twice and used throughout this study. The conditions for cell culture and plaque assay have been reported (Ortin et al., 1980; Tobita et al., 1975).

The β-actin cDNA clone pHF5 (Gunning et al., 1983) was kindly provided by L. Sastre. The cDNA clones specific for α-actin (Hanauer et al., 1983), α-tubulin (Cowan et al., 1983), β-tubulin (Valenzuela et al., 1983) and vimentin (A. Alonso & J. L. Jorcano, unpublished results) were a gift from J. L. Jorcano. The origin of M1-specific (pBK7/2) and HA-specific (pSVa970) cDNAs has been described (Ortin et al., 1983; Portela, 1986).

Isolation and analysis of RNA. Nuclear and cytoplasmic fractions were prepared from infected or mock-infected COS-1 cells as described (Ortin & Doerfler, 1975), and cytoplasmic RNA was obtained by incubation with protease K (200 µg/ml) in a buffer containing 100 mM-Tris-HCl, 12 mM-EDTA, 150 mM-NaCl, 1% SDS, pH 7.5, and extraction with a phenol–chloroform–isoamyl alcohol–hydroxyquinoline (50:50:1:0.1) mixture. Alternatively, total cell RNA was obtained by the guanidinium thiocyanate method (Chirgwin et al., 1979). In some experiments, poly(A)⁺ RNA was selected by oligo(dT)-chromatography (Aviv & Leder, 1972). In those cases, recovery from the columns was checked by including a ³H-labelled riboprobe derived from clone pSVa970, which contains an encoded poly(A) tract (Valc{trceil et al., 1991). Identical amounts of RNA were electrophoresed under denaturing conditions (Lehrach et al., 1982), and hybridized to probes specific for the genes indicated above. The labelled probes were generated from the cloned inserts by the random primer method (Feinberg & Vogelstein, 1983) and hybridized to probes specific for the genes indicated above. The labelled probes were generated from the cloned inserts by the random primer method (Feinberg & Vogelstein, 1983), using T7 DNA polymerase. Alternatively, cDNA probes were synthesized as described (Sambrook et al., 1989) using uninfected cell poly(A)⁺ RNA as the template. The autoradiographic signals were quantified by microdensitometry.

Protein labelling and analysis. Cells were grown in 24-well plates with 1 ml of DMEM supplemented with 5% foetal bovine serum. For protein labelling, infected or mock-infected cells were incubated for 1 h in methionine-free DMEM and labelled for 1 h in DMEM containing 200 µCi [³⁵S]methionine per ml. Labelled cells were washed with DMEM and resuspended in 100 µl of lysis buffer. Two-dimensional gel electrophoresis was performed as described by O’Farrell (1975), with minor modifications (Santarén & Bravo, 1987). Briefly, the first dimension separation to resolve acidic proteins (isoelectric focusing, IEF) was carried out in 230 x 2.3 cm, 4% polyacrylamide gels (24 x 24 cm). Gels were processed for fluorography (Laskey & Mills, 1975), dried and exposed at −70 °C for various periods of time. Approximately 1 x 10⁶ TCA-insoluble c.p.m. were applied per gel. For quantification, the fluorograms were scanned using a Molecular Dynamics computing densitometer (model 300A) and the gel regions corresponding to the spots were cut out and processed as described previously (Bravo et al., 1982).

Results and Discussion

For the analysis of the metabolism of M1 and M2 mRNAs, we used the β-actin gene as an internal standard for mRNA recovery in the determination of their half-lives (Valcárcel et al., 1991). The results obtained suggested the rapid degradation of β-actin mRNA during influenza virus infection. Thus, a systematic study of the stability of cellular mRNAs and the cellular protein synthesis was undertaken.

Degradation of cellular mRNAs in influenza virus-infected cells

To measure the half-life of cellular mRNAs in influenza virus-infected cells, cytoplasmic RNA was isolated at several times after high multiplicity infection and the relative concentration of α- and β-actin, α- and β-tubulin and vimentin mRNAs, as a representative set of cellular mRNAs, was determined by blot hybridization. As an internal control of virus infection, the accumulation of matrix protein (M1) mRNA was also determined. The results, including the quantification of the bands by densitometry, are shown in Fig. 1. The decrease of cellular mRNA concentration correlates with the accumulation of viral mRNAs, represented by M1 mRNA. Some of them, like β-actin mRNA, decayed very rapidly: its concentration is reduced to 50% at a time when M1 mRNA is still undetectable. Others, like α-tubulin mRNA, appeared to be more stable. Essentially the same data were obtained when poly(A)⁺ RNA was used to perform the analysis (data not shown).

One possibility to explain these results would be that cellular mRNAs are relocalized in the cell after infection in such a way that they are no longer available for extraction under the usual conditions of nucleocytoplasmic fractionation. To test this hypothesis, total cell RNA was extracted at several times after influenza virus infection and the relative concentrations of the cellular mRNAs indicated above were determined. The results, presented in Fig. 2, are indistinguishable from those shown in Fig. 1. Thus, it is concluded that cellular mRNA concentration decreases in the course of influenza virus infection.

Since the probes used correspond to cytoskeletal protein genes, it could be argued that the decrease in mRNA concentration might not be a general phenomenon but affects only a subclass of cellular mRNAs. To check this possibility, a cDNA probe was synthesized using uninfected cell poly(A)⁺ RNA as the template and the blots were rehybridized. The results obtained were consistent with those presented in Fig. 1 and 2 (data not shown). In addition, the kinetics of decay for cellular mRNAs were assayed in influenza virus-infected MDCK cells by blot hybridization using as probes β-tubulin, vimentin and M1 cDNAs. As shown in Fig. 3, the concentration of both cellular mRNAs decreased rapidly, in a manner indistinguishable from that obtained for infected COS-1 cells (see Fig. 2). Taken together, these results confirm and extend those reported earlier (Inglis, 1982) but are in disagreement with some of the data presented by Katze & Krug (1984).
Fig. 1. Decay of cellular mRNAs in the cytoplasm of influenza virus-infected COS-1 cells. COS-1 cell cultures were infected with the A/Victoria/3/75 strain of influenza virus at an approximate multiplicity of 10 p.f.u. per cell. At the times after infection indicated in the figure, cytoplasmic fractions were obtained, from which total RNA was extracted, as indicated in Methods. Samples (20 µg) of RNA, as determined by absorbance determinations and checked by trial agarose gel electrophoresis, were run in formaldehyde-agarose gels, transferred to nylon membranes and hybridized to the probes indicated in the figure. Appropriate exposures of the films (from 9 h to 2 days, depending on the probe used) were quantified by microdensitometry and the values are presented as percentage of the maximum values.

The decrease in concentration of cellular, cytoplasmic mRNAs could be explained by an inhibition of their synthesis and/or nucleocytoplasmic transport coupled to an intrinsic instability. Since the synthesis and nucleocytoplasmic transport of cellular mRNAs is inhibited in influenza virus-infected cells (Katze & Krug, 1984), it remains to be established whether they are particularly unstable in the cytoplasm. The half-life of the cytoplasmic mRNAs under study was determined by inhibition of transcription with actinomycin D and blot hybridization of RNAs isolated at several times thereafter. As shown in Fig. 4, the cytoplasmic mRNAs specific for α- and β-actin, α- and β-tubulin and vimentin were stable in the time range considered. Therefore, it is proposed that infection with influenza virus induces a specific degradation of cellular mRNAs.
Fig. 3. Decay of total cellular mRNAs in influenza virus-infected MDCK cells. MDCK cell cultures were infected and total cell mRNA was extracted as indicated in the legend to Fig. 2. Samples (20 μg) of RNA were electrophoresed, blotted and probed as indicated in the legend to Fig. 1. Exposure times ranged from 2 to 8 days, depending on the probe.

How could infection by influenza virus mediate the degradation of cellular mRNAs? Several pieces of evidence suggest that the stability of mRNAs is connected to the integrity of their poly(A) tracts: polyadenylation of normally non-polyadenylated mRNAs, such as histone mRNAs, leads to an increase in their stability in vivo or in vitro; removal of the poly(A) tract kinetically precedes the degradation of the mRNA body; elongation of the poly(A) tract correlates with the stabilization of certain mRNAs, like human growth hormone mRNA (Huez et al., 1978; Mercer & Wake, 1985; Paek & Axel, 1987; reviewed in Bernstein & Ross, 1989; Braverman, 1989). In turn, the stability of the poly(A) tract appears to be related to its interaction with the poly(A)-binding protein (PABP), which would increase its resistance to nucleases (Bernstein et al., 1989). There are several possible mechanisms by which influenza viruses could induce mRNA degradation: (i) the virus could encode a nuclease able to reduce the half-life of mRNAs; (ii) the virus could activate a pre-existing cellular nuclease; (iii) the virus could alter the normal mechanism for protection of mRNAs and hence render them more accessible to nucleases. An attractive hypothesis from the last possibility would be that a virus-induced protein is able to interact with PABP, thereby inhibiting its function in mRNA protection.

How would viral mRNAs escape such a general mRNA degradation? The available data suggest that viral mRNA stability is rather limited: the half-life of both M1 and M2 mRNAs is 2.7 h (Valcárcel et al., 1991) and the kinetics of viral mRNA accumulation in infection indicate a similar stability for many of them (Hatada et al., 1989; and our unpublished results). Hence, the possibility exists that the destabilization of mRNAs is a general phenomenon affecting both cellular...
mRNA degradation

Fig. 5. Kinetics of cellular protein synthesis in influenza virus-infected cells. Cell cultures were infected with influenza virus as indicated in the legend to Fig. 1. At the times indicated in the figure, the cultures were pulse-labelled for 1 h with [3S]methionine and total cell extracts were prepared as described in Methods. Samples of 10^6 c.p.m. of each extract were analysed by two-dimensional gel electrophoresis. Only the area of interest in the gel is shown. The arrows indicate the spots corresponding to β-actin (a), α-tubulin (α.t), β-tubulin (β.t) and vimentin (v). The radiactivity associated with each spot was quantified by liquid scintillation or by microdensitometry of the corresponding films.

and viral mRNAs, much in the way proposed for herpes simplex virus (Kwong & Frenkel, 1987; Kwong et al., 1988).

Inhibition of cellular protein synthesis in influenza virus-infected cells

To ascertain the degree to which the synthesis of the cellular proteins encoded by the genes under study was inhibited, mock-infected or influenza virus-infected cells were pulse-labelled at several times after high multiplicity infection. The proteins present in total cell extracts were resolved by two-dimensional gel electrophoresis and the identification of the spots corresponding to β-actin, α- and β-tubulin and vimentin was carried out by using some of the two-dimensional gel protein databases available (Celis et al., 1990). The radioactive label associated with such spots was quantified as described in Methods. The results, presented in Fig. 5, indicate that the synthesis of cellular protein showed kinetics of decay that paralleled that of cellular mRNA decay (Fig. 1 and 2). Quantification of several other anonymous spots indicated that the overall rate of protein synthesis inhibition during infection was indistinguishable from that shown in Fig. 5 (data not shown). These results suggest that the observed cellular mRNA degradation might be responsible for the inhibition of cellular protein synthesis after influenza virus infection. The alternative possibility, i.e. that an inhibition in protein synthesis causes the degradation of cellular mRNAs is unlikely, since mRNA decay requires protein synthesis (Braverman, 1989).

A direct inhibition of cellular protein synthesis in influenza virus-infected cells has been proposed (Katze & Krug, 1984; Katze et al., 1986a). The mechanism suggested for such inhibition would involve the impairment of a cellular initiation factor dispensable for influenza virus mRNA translation and the takeover of the translation machinery by the influenza virus mRNAs, which are particularly efficient. The affected initiation factor would be other than eIF-2, since its phosphorylation is prevented during influenza virus infection (Katze et al., 1986b, 1988). In fact, the lack of eIF-2 phosphorylation might be the consequence of an influenza virus activity similar to adenovirus VA RNA, which would avoid a generalized protein synthesis inhibition (Katze et al., 1988). It is difficult to ascertain the reasons for the partial discrepancies observed between the results presented here and those reported earlier (Katze & Krug, 1984). One possible explanation might be that, under the very high multiplicity used before, a virion-induced alteration of the host cell is produced which leads to cell protein synthesis inhibition at an early phase in the infection, before cellular mRNA degradation is observed. Our results, obtained under single-cycle infection but at lower multiplicity, do not rule out the existence of a direct cell protein synthesis inhibition, but are better explained by a model that would include the degradation of mRNAs in the infected cells. Under these circumstances, viral mRNAs would take over the cellular protein synthesis machinery, since they are synthesized at much faster rates than the cellular mRNAs and their translation is particularly efficient (Katze et al., 1986b).

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