Regulated M1 mRNA splicing in influenza virus-infected cells

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Influenza virus RNA segment 7 generates three poly(A)† RNAs, M1 mRNA, M2 mRNA and mRNA3, the last of which has almost no coding capacity; M2 mRNA and mRNA3 derive from M1 mRNA by removal of a single intron. The kinetics of M1 and M2 mRNA accumulation in the cytoplasm of productively infected cells were studied by means of a quantitative RNA protection assay; the ratio of M2 mRNA to M1 mRNA increased 2.7-fold during the course of infection. To analyse the basis for this change, the kinetics of M1 and M2 mRNA synthesis and nuclear accumulation, their stability and nucleocytoplasmic transport were studied. Under the experimental conditions used, the synthesis of segment 7-specific RNA showed a peak at 4 h post-infection and continued later at a slower rate. The half-lives of M1 and M2 mRNAs were indistinguishable (2.7 h for M1 mRNA and 2.7 h for M2 mRNA) and the kinetics of nucleocytoplasmic transport in vivo or in vitro showed no preference for either mRNA early or late in infection. Consequently, regulation at the level of mRNA splicing is proposed. Using the mRNA synthesis and stability data, a simulation was performed to predict the change in splicing efficiency required to account for the mRNA accumulation results. The best fit was obtained when splicing efficiency changed about 20 times during a period in which viral gene expression was maximal.

Introduction

The genome of influenza type A virus consists of eight ssRNA segments of negative polarity, encoding at least 10 polypeptides (for a review see Lamb, 1983). RNA segment 7 has the potential to encode two polypeptides, M1 and M2 (Allen et al., 1980), each translated from a separate mRNA (Inglis & Brown, 1981; Lamb et al., 1981). Protein M1 is the most abundant polypeptide in the virion, is located underneath the virus membrane and interacts with both the viral ribonucleoproteins (Zvonarjev & Ghendon, 1980; Ye et al., 1987, 1989) and the membrane (Gregoriades & Frangione, 1981; Ye et al., 1987). A comparison of the M1 protein gene from several virus strains indicates that it is more conserved than other viral genes (Ortin et al., 1983), probably due to its interaction with several viral and/or cellular components during virus morphogenesis. Protein M2 is an integral membrane protein that accumulates at the plasma membrane of infected cells (Lamb et al., 1985), with a conserved, externally exposed NH2-terminal amino acid tail and a cytoplasmic C-terminal domain (Lamb et al., 1985; Zebedee et al., 1985). It is palmitoylated in some virus strains ( Sugrue et al., 1990 a) and, although it is abundant on the cell surface, can only be detected in small quantities in the virion (Zebedee & Lamb, 1988). The fact that mutations leading to amantadine resistance map to its coding region (Hay et al., 1985) and that there is genetic interaction between M2 and M1 cistrons (Zebedee & Lamb, 1989) suggests a role for M2 protein in morphogenesis. On the other hand, it has been proposed that the M2 protein could prevent a low pH-induced conformational change of the haemagglutinin (HA) molecule in its maturation by the exocytic pathway (Sugrue et al., 1990 b).

The mRNA encoding the M1 protein is collinear with RNA segment 7, whereas M2 mRNA is a spliced product of M1 mRNA (Lamb et al., 1981). The first exon encodes nine amino acids identical to those of the M1 protein, but the second exon uses a different reading frame. An alternative donor site generates a third RNA species, mRNA3, with very little coding capacity (Inglis & Brown, 1981). These splicing events are probably carried out in the nucleus by the cellular machinery because the splice junctions contain the consensus sequences (Mount, 1982). The splicing process also takes place when M1 mRNA is transcribed from simian virus 40 (SV40) recombinants containing RNA segment 7 genetic information; mRNA3 is produced from a late SV40 recombinant (Lamb & Lai, 1982) and M2 mRNA is synthesized from an early SV40 construct (our unpublished results). The basis for this difference is unknown at present, but may reflect the different
structures at the 5' and 3' ends of the transcripts generated by either promoter.

The steady-state level of M2 mRNA in infected cells is regulated in such a way that it remains a fraction of that of M1 mRNA (Lamb et al., 1981). This is not unexpected because both M1 and M2 proteins are required for virus replication. Furthermore, restricted expression of M1 protein and a concomitant increase in M2 protein levels is observed in abortively infected L cells (Inglis & Brown, 1984) and in several cultured mouse embryo brain cells (Bradshaw et al., 1990), suggesting an important role for this regulation in the virus life cycle. The level at which this regulation takes place is unclear, but the observation that inhibition of protein synthesis during infection abolishes the accumulation of M2 mRNA (Inglis & Brown, 1984) suggests that a viral product is involved. The modulation of the steady-state levels of alternatively spliced products is not exclusive to influenza virus RNA segment 7; it occurs also for RNA segment 8, encoding the NS1 and NS2 proteins (Lamb & Lai, 1980), and for collinear and spliced mRNAs in retroviruses (Varmus, 1988; Katz et al., 1988; Cullen & Greene, 1989).

In this report, we have examined the synthesis, accumulation, stability and nucleocytoplasmic transport of M1 and M2 mRNAs in cells infected with influenza virus. The results indicate that the ratio of steady-state levels of M1- and M2-specific mRNAs change during the infection cycle, and suggest that the efficiency of M1 mRNA splicing is responsible for this temporal regulation.

Methods

Viruses and cells. The A/Victoria/3/75 (H3N2) strain of influenza A virus was cloned by two cycles of plaque isolation and used throughout this study. The MDCK cell line was obtained from the American Type Culture Collection. The conditions for cell culture, virus growth and plaque assay were as described (Ortin et al., 1980).

Gene cloning. RNA segment 7 was cloned into the pBSV9 vector by oriented cDNA synthesis, using in vitro mRNA as template and a virus-specific oligodeoxyribonucleotide primer for second-strand synthesis, as described previously (Portela et al., 1985). The BgII–ClaI (positions 136 to 868) and the NheI–BgII (positions 1 to 141) fragments were inserted into pGEM4 vectors (Promega Biotec), generating recombinants pG7BC and pG7NB respectively. The 1762 of the influenza virus HA gene.

RNase protection assay. Labelled riboprobes were synthesized on linearized pG7NB plasmid with Sp6 RNA polymerase in the presence of $[^32P]CTP$ (5 x 10³ c.p.m./pmol) (Melton et al., 1984). For the RNase protection assay, approximately 1 fmo of segment 7-specific RNA was mixed with a 10-fold excess of probe, ethanol-precipitated and resuspended in 80% formamide, 0-4 M-NaCl, 1 mM-EDTA, 40 mM-PIPES pH 6-4. After heating for 5 min at 85 °C, the mixture was hybridized for between 12 and 24 h at 50 °C. Under these conditions, hybridization equilibrium was obtained for both M1 and M2 mRNAs, as determined in kinetic experiments (data not shown). Samples were diluted into 0-3 M-NaCl, 5 mM-EDTA, 10 mM-Tris–HCl pH 7-5 and incubated for 1 h at 37 °C with 10 μg/ml RNase A. Digestion products were analysed by electrophoresis on sequencing gels and autoradiography. Controls to ensure riboprobe excess and to check the linearity of film exposure were included in each experiment. Protected bands were quantified by microdensitometry.

Filter hybridizations. Plasmid pBK7/2 DNA (Ortin et al., 1983) or pBR322 DNA was denatured and fixed to nitrocellulose filters. After prehybridization for 12 h at 65 °C in a buffer containing 5 x SSC, 5 x concentrated Denhardt's mixture, 0-5% SDS and 200 mg/ml yeast RNA, they were incubated with appropriate amounts of $[^32P]$labelled poly(A)+ RNA for 24 h at 65 °C. Filters were washed twice in 2 x SSC, 0-5% SDS and twice in 0-1 x SSC, 0-5% SDS for 30 min at 65 °C, and filter-bound radioactivity was determined by liquid scintillation.

Nucleocytoplasmic transport. MDCK cells were infected at an m.o.i. of 10 to 20 p.f.u./cell. Nuclei were isolated and nucleocytoplasmic RNA transport was assayed as described by Schroeder et al. (1989). In brief, cells were cooled on ice, washed and scraped off the plates into 10 mM-Tris–HCl pH 7-5, 1 mM-MgCl₂, 1 mM-EDTA. After Dounce homogenization in the same buffer including 1 mM-PMSF, the suspension was made 50% in sucrose and centrifuged on a 60% sucrose cushion in the same buffer for 1 h at 4 °C at 32000 r.p.m. in an SW40 rotor. Nuclei were resuspended in transport medium (25 mM-Tris–HCl pH 7-5, 250 mM-sucrose, 2 mM-MgCl₂, 0.5 mM-CaCl₂, 0.3 mM-MnCl₂, 25 mM-KCl, 5 mM-spermidine, 5 mM-2-mercaptoethanol and 300 μg/ml yeast RNA). Aliquots (840 μl) were mixed with 160 μl of eI. ATP-regenerating system (15-6 mM-ATP, 31-2 mM-Na₂HPO₄, 31-2 mM-phosphoenolpyruvate and 218 U/ml pyruvate kinase) or water. The kinetics of RNA transport were studied at 30 °C. Aliquots of the suspension were collected and cooled on ice at various times during incubation, and centrifuged for 4 min at 10000 g at 0 °C. RNA was extracted from the supernatants and purified by oligo(dT)–cellulose chromatography. Recovery was checked as described above and the M1 and M2 mRNAs were quantified by RNase protection.
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Results

Kinetics of M1 and M2 mRNA synthesis and accumulation

The kinetics of influenza virus mRNA synthesis have been reported (Enami et al., 1985; Shapiro et al., 1987). We have confirmed these results, to be certain that in the experimental system used (influenza virus strain A/Victoria/3/75 and MDCK cells) the kinetics of M1 and M2 mRNA synthesis were similar to that reported. Poly(A)⁺ RNA, pulse-labelled at various times after infection, was hybridized to cloned segment 7 DNA and the hybrids were quantified by liquid scintillation. As shown in Fig. 1, a large fraction of the segment 7-specific mRNA is synthesized around 4 h post-infection (p.i.), but the synthesis continues at a slower rate later in the infection. Since the results were obtained using full-length cDNA, it is not possible to ascertain which fraction of the newly synthesized segment 7-specific mRNA corresponds to M1 or M2 mRNAs.

To study the accumulation of M1 and M2 mRNAs, an RNase protection assay was set up that used as probe a synthetic RNA including positions 1 to 141 of the RNA segment 7 sequence. M1 mRNA should protect the full-length virus-specific sequence in the probe, whereas M2 mRNA would protect only the first 51 nucleotides, from the viral 5' terminus to the splicing donor site (Lamb et al., 1981; Lamb & Lai, 1982). mRNA3 would protect 11 nucleotides of the probe but the hybrid would not be stable under the hybridization conditions used. Primer extension experiments indicated that mRNA3 was produced throughout the infection in roughly the same amounts as M2 mRNA (data not shown), but since these assays were not quantitative this approach was not pursued further. The proportion of M1 mRNA to M2 mRNA was determined by the protection assay described above using excess labelled probe and allowing the hybridization reaction to reach equilibrium. The results, shown in Fig. 2, indicate that at early times after infection most of the segment 7 transcripts corresponded to M1 mRNA, and that the proportion of M2 mRNA increased later. M2 mRNA accounted for 3% of segment 7 mRNA at 3 h p.i. and 8-4% at 9 h p.i. on a molar basis. This means the ratio of M1 mRNA to M2 mRNA changes 2.7-fold. These results were obtained reproducibly in several independent experiments involving different RNA preparations and using different host cells (MDCK, COS-1 and CEF). Similar kinetic effects were observed when a riboprobe specific for the splicing acceptor site was used, suggesting that the change observed for M2 mRNA may also occur for the mRNA3 alternative splice product (data not shown).

Stability of M1 and M2 mRNAs in MDCK-infected cells

The change in the ratio of M1 mRNA to M2 mRNA during the infection cycle could be explained by a difference in the stability of the corresponding mRNAs. To check that possibility, MDCK cells were infected with influenza virus and the synthesis of RNA was inhibited by the addition of actinomycin D at 5 or 6 h p.i. Since the synthesis of influenza virus mRNA is strictly dependent on cellular mRNA synthesis in the cell nucleus (Scholtissek & Rott, 1970), the inhibition of the latter leads to a blockade of viral transcription. The amount of M1 or M2 mRNA present in the cytoplasm at various times after the addition of inhibitor was determined by the RNase protection assay described in Methods. The results of such an experiment are shown in Fig. 3. The average values obtained for the half-lives of M1 mRNA (2.73 ± 0.83 h) and M2 mRNA (2.70 ± 0.46 h) in three independent experiments were indistinguishable, and hence a differential stability cannot account for the changes in mRNA accumulation observed. Furthermore, none of the experimental pairs of values obtained for the half-lives in each experiment would account for the kinetics of accumulation observed.

Nucleocytoplasmic transport

Another possibility to explain the change in the ratio of M1 mRNA to M2 mRNA accumulation during infec-
tion is to assume different kinetics for the nucleocytoplasmic transport of M1 or M2 mRNAs early and late in the infection. If this were the case, preferential accumulation of M2 mRNA in the nucleus would be expected at early times in infection. Alternatively, if late M1 mRNA transport was impaired, M1 mRNA should accumulate preferentially in the nucleus at late times in the infection. Moreover, since the absolute amounts of segment 7-specific mRNAs are 10-fold greater in the cytoplasm than in the nucleus, this preferential accumulation should be very large. As shown in Fig. 4, there is no preferential accumulation of M2 or M1 mRNA in the nucleus early or late in the infection, respectively, and hence these results are not compatible with an unbalanced nucleocytoplasmic transport being the cause for the observed differential M1 and M2 mRNA cytoplasmic accumulation. In fact, quantification of two independent experiments indicated that the ratio of M2 mRNA to M1 mRNA increased 2.3-fold in the nucleus of the cells between 3 and 9 h p.i.

The rate of nucleocytoplasmic transport was directly measured by pulse–chase experiments in which cell cultures were pulse-labelled with [3H]uridine for 30 min at 3 or 7 h.p.i. and chased for different times thereafter. Nuclear and cytoplasmic fractions were obtained and the
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Fig. 4. Kinetics of M1- and M2-specific nuclear RNA accumulation. Cell cultures were infected as described in the legend to Fig. 2 and nuclear RNA was isolated at 3, 5, 7, and 9 h p.i. (lanes 1 to 5). Samples of purified poly(A)+ RNA containing equal amounts of segment 7-specific RNA (approximately 1 fmol as determined by previous dot-blot hybridization) were hybridized to riboprobe 7NB to determine the proportion of M1- and M2-specific mRNAs, as described in the legend to Fig. 2. To detect M1- and M2-specific protection bands, the sample was exposed for 4 h and 47 h, respectively. The amount of RNA used was equivalent to 2.4 x 10^6, 1.8 x 10^6, 1.4 x 10^6, 0.6 x 10^6 and 1.1 x 10^6 cells, lanes 2 to 6 respectively. Lane 1, RNA from mock-infected cells.

segment 7-specific radioactivity was determined by filter hybridization to either a full-length cDNA or an intron probe. The former should detect both M1 and M2 mRNAs, whereas the latter should hybridize only to M1 mRNA. The results showed similar kinetics for the transport of M1 mRNA or M1 + M2 mRNA early or late in infection (data not shown). Since these results suffered from the low sensitivity of M2 mRNA determination, they were confirmed by in vitro nucleocytoplasmic transport assays, as indicated below.

**Quantitative analysis of the data**

The results regarding the kinetics of segment 7-specific RNA synthesis and the stability of cytoplasmic M1 and M2 mRNA can be used to predict their cytoplasmic accumulation on the assumption, supported by the results presented in Fig. 4 and 6, that the kinetic constant of nucleocytoplasmic transport does not change during infection. To make such a prediction, a value for the efficiency of M1 mRNA splicing has to be assumed. From the accumulation of M1 and M2 mRNAs 3 h p.i. (Fig. 2), the initial value of splicing efficiency can be estimated to be 3.1%, i.e. one in every 32 M1 mRNA molecules would be spliced to yield M2 mRNA. The ratio of M1 mRNA to M2 mRNA accumulation was predicted at different times after infection, using an initial 3.1% splicing efficiency and assuming several possible patterns of splicing efficiency changes in the course of virus infection. Some of these predictions as well as the experimental values (curve e) are shown in Fig. 5(a). The simplest assumption, i.e. a splicing efficiency constant and equal to 3.1% (Fig. 5b, curve A), does not reflect the experimental data (Fig. 5a, curve a). In curve B, a steady increase in splicing efficiency (8%/h) was used. As a third possibility, the efficiency of
M1 mRNA

\[ \text{M1 mRNA} \]

\[ \text{M2 mRNA} \]

Fig. 6. \textit{In vitro} nucleocytoplasmic transport. Nuclei were isolated from infected MDCK cells at 3 (lanes 1 to 5) and 7 (lanes 6 to 10) h p.i. The transport of M1 and M2 mRNAs was studied by incubation in transport buffer or water. At 0 min (lanes 1, 3, 6 and 8), 5 min (lanes 4 and 9) and 10 min (lanes 2, 5, 7 and 10), nuclei were centrifuged, the RNA present in the supernatant was purified and the accumulation of M1 and M2 mRNAs was determined by a quantitative RNase protection assay, as described. The RNA which accumulated in the supernatant in the absence of the ATP-regenerating system was 5 to 10\% that of the maximal transported RNA, confirming that active transport rather than leakage occurs. The proportion of nuclear RNA that was transported \textit{in vitro} was measured and ranged between 12 and 20\%, in accordance with published results (Schroeder \textit{et al.}, 1989). Experiments with longer incubation times showed that maximal transport was achieved by 10 min (data not shown). The bands specific for M2 RNA were exposed five times longer than those specific for M1 RNA.

Splicing was increased by a fixed proportion (40\%) of the previous one (curve C). In neither case did the predicted ratio of M1 mRNA to M2 mRNA accumulation conform to the experimental results (Fig. 5a, curves b and c). Finally, using a trial-and-error approach, an optimal pattern for the splicing efficiency variation was obtained that led to a good fit with the experimental values (curve d). It is a non-linear function of the time after infection, with an abrupt change around 5 h p.i. According to this optimal prediction, the efficiency of splicing of M1 mRNA would change about 20 times during infection and the maximal change would be concomitant with maximal viral gene expression.

Direct support for the above prediction could be obtained by analysis of the M1 and M2 mRNAs transported \textit{in vitro} from nuclei isolated early (3 h p.i.) or late (7 h p.i.) in the infection cycle. This assay allows the detection of nuclear RNAs that have been processed \textit{in vivo} and were ready to be transported at the time the nuclei were isolated. The ratio of M1 mRNA to M2 mRNA transported \textit{in vitro} is therefore a reflection of the efficiency with which M1 mRNA was spliced to produce M2 mRNA at that particular time. The results of this assay are shown in Fig. 6. The kinetics of nucleocytoplasmic transport confirmed the results obtained \textit{in vivo} and, more interestingly, the plateau levels indicated that the molar ratio of M2 mRNA to M1 mRNA consistently increased 3-5-fold between 3 and 7 h p.i. Since the mRNAs transported \textit{in vitro} from nuclei isolated at 7 h p.i. were spliced \textit{in vitro} between 6 and 7 h p.i., these values are in reasonable agreement to those predicted from the synthesis, accumulation and decay data (Fig. 5), and strongly support the notion that M1 mRNA splicing is regulated during influenza virus infection.

**Discussion**

One of the ways in which gene expression can be regulated is by the splicing of mRNA precursors. Although most pre-mRNAs are constitutively spliced to remove all their introns, there are many examples in which the efficiency of splicing at particular signals is reduced. This situation leads to alternative splicing, i.e. two or several mRNAs can be produced from a single pre-mRNA, containing different combinatorial sets of exons. This situation is particularly frequent for various families of structural proteins, like tropomins and tropomyosins, that are generated from single genes by this mechanism (Breitbart \textit{et al.}, 1987). Since some of the family members are tissue-specific, trans-acting factors might be involved in regulation, probably by interaction with mutually exclusive secondary structures (cis-acting elements) present in these pre-mRNAs (Smith & Nadal-Ginard, 1989; Goux-Pelletan \textit{et al.}, 1990). Another well studied example is the sex determination process in drosophila, in which a cascade of genetic interactions, known at least in part to involve trans-regulation of pre-mRNA splicing, has been identified (Nagoshi \textit{et al.}, 1988; Hodgkin, 1989; Baker, 1989; Nagoshi & Baker, 1990).

In viral systems, alternative splicing is a mechanism whereby genetic information can be tightly packed into the limited size of viral genomes. Thus, several proteins are expressed from complex transcription units in adenoviruses (Green, 1986) and the appropriate level of gag versus env gene expression is obtained in simple retroviruses. Whether trans-acting factors are involved in the regulation or cis-features dictate a constitutive inefficiency of the process is not clear at present (Katz \textit{et al.}, 1988; Katz & Škalka, 1990). In the case of complex retroviruses like human immunodeficiency virus type 1, \textit{rev} protein regulates the cytoplasmic accumulation of viral unspliced and single-spliced mRNAs in relation to doubly spliced mRNA, which encodes viral regulatory proteins including the \textit{rev} protein (Feinberg \textit{et al.}, 1986; Green & Zapp, 1989). Increased nucleocytoplasmic transport of mRNAs containing a \textit{rev}-responsive sequence (REE or CAR) has been reported (Emerman \textit{et al.}, 1989; Malim \textit{et al.}, 1989; Dayton \textit{et al.}, 1989), perhaps related to a blockade in splicesomal resolution for these pre-mRNAs in the absence of \textit{rev} protein (Chang & Sharp, 1989).
In influenza viruses, each of the two smaller RNA segments encode two proteins, the largest of which is translated from the collinear full-length transcript, whereas the smallest one is encoded by a spliced mRNA product (Lamb, 1983). It is apparent that an adequate balance between spliced and unspliced mRNAs has to be achieved, since both gene products (M1 and M2 in segment 7, and NS1 and NS2 in segment 8) are essential for virus replication. In fact, a correlation exists between unbalanced expression of segment 7 and 8 gene products and abortive infections (Inglis & Brown, 1984; Bradshaw et al., 1990).

The implication of cis regulatory elements in the control of mRNA levels has been suggested by in vitro splicing experiments involving segment 8 RNA (Plotch & Krug, 1986). In spite of being able to direct the formation of spliceosomes in vitro, segment 8 RNA is extremely inefficient in the splicing reaction (Agris et al., 1989; Plotch & Krug, 1986). On the other hand, lack of accumulation of NS2 or M2 mRNAs in the absence of protein synthesis suggests a role for trans-acting factors in regulation (Inglis & Brown, 1984). Furthermore, a viral mutant showing reduced accumulation of NS2 mRNA can be complemented by infection with wild-type virus (Smith & Inglis, 1985).

As a first step in the study of the process, we have undertaken an analysis of M1 and M2 synthesis, accumulation, stability and nucleocytoplasmic transport. The results obtained for cytoplasmic accumulation (Fig. 2) indicate an increase in the ratio of M2 mRNA to M1 mRNA as infection proceeds. In principle, there are three possibilities to explain these results: (i) differential stability of M1 and M2 mRNAs, (ii) a defect in M2 or M1 mRNA nucleocytoplasmic transport early or late in infection, respectively, and (iii) a change in the splicing efficiency of M1 mRNA over the course of infection. The data presented in Fig. 3 rule out the first possibility, since the half-life of either mRNA is indistinguishable throughout the infection cycle. Evidence from several experimental approaches (Fig. 4 and 6) makes the second possibility very unlikely. Therefore, we conclude that splicing of influenza virus RNA segment 7 is regulated during the infection cycle. This conclusion is strongly supported by direct estimation of the ratio of spliced mRNA to unspliced mRNA leaving the nucleus early or late in infection (Fig. 6).

It is possible that, as suggested for other experimental systems like influenza virus segment 8 (Plotch & Krug, 1986; Agris et al., 1989) or ~-tropomyosin (Smith & Nadal-Ginard, 1989), an unusual RNA structure renders segment 7 RNA a poorly spliceable substrate. In fact, both the presumable branch point and the acceptor site sequences in RNA segment 7 are predicted to form stable stem structures (data not shown). However, it seems unlikely from the experimental data presented that regulation is mediated solely by cis-acting elements. The change in the pattern of splicing efficiency proposed in Fig. 5 might suggest a viral trans-acting factor as a mediator of this regulation, in accordance with previous results (Inglis & Brown, 1984; Smith & Inglis, 1985).

In conclusion, influenza virus segment 7 RNA splicing might involve not only an important regulatory point in the viral infection cycle, but might be a simple biological model to study complex regulatory phenomena.

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