Stimulation of Sendai virus C′ protein synthesis by cycloheximide

Kailash C. GUPTA* and Etsuro ONO†

*Department of Immunology/Microbiology, Rush Medical College, 1653 West Congress Parkway, Chicago, IL 60612, U.S.A.

The polycistronic Sendai virus P/C mRNA is translated into five proteins (P, C′, C, Y1 and Y2) from distinct start sites in virus-infected cells. The translation mechanism(s) of these proteins from two overlapping open reading frames in the P/C mRNA are poorly understood [Gupta, Ono and Xu (1996) Biochemistry **35**, 1223–1231]. While investigating the initiation mechanism of C′ from an ACG start site, we found that C′ synthesis was resistant to inhibitors of peptide chain elongation such as cycloheximide (CHX) and anisomycin, but not to pactamycin (an inhibitor of chain initiation) or puromycin (a peptide chain terminator). Moreover, low levels (less than 30 μg/ml) of CHX significantly stimulated C′ synthesis. Whereas C′ synthesis was stimulated, synthesis of the P and C proteins, which are translated from the same mRNA, decreased by more than 95 %. Stimulation of C′ synthesis by CHX is not related to its initiation at an ACG codon. Mutation of ACG to alternative start sites had no effect on the CHX-stimulated C′ synthesis. Similarly, C′ synthesis was preferentially stimulated when Sendai virus-infected cells were exposed to hypotonic growth medium. These results suggest that the P/C mRNA may exist in at least two reversible conformations: whereas one conformation allows synthesis of the P and C proteins, the alternative conformation allows synthesis of the C′ protein. It might be that low concentrations of CHX somehow increase the alternative conformation, which increases C′ synthesis. The C′ protein synthesis is reminiscent of the synthesis of stress-related proteins. Perhaps Sendai virus has evolved a novel mechanism to express both non-stress-related and stress-related proteins from the same mRNA.

INTRODUCTION

Sendai virus is a prototypic paramyxovirus [1]. Paramyxoviruses are negative-strand RNA viruses that replicate in the cytoplasm of host cells. As in other paramyxoviruses, the Sendai virus P gene has evolved a highly unusual and compact coding strategy [2]. First the P gene is transcribed into at least two mRNA species (P/C and V/C) by virtue of a nucleotide insertion by the viral RNA polymerase during transcription [3], and secondly both the mRNA species are functionally polycistronic, encoding several proteins (P, V, C′, C, Y1 and Y2) from two overlapping reading frames (Figure 1). Moreover, synthesis of the C′ protein is initiated from a non-AUG codon [4,5]. The mechanism(s) by which various proteins from these mRNAs are synthesized are poorly understood [6,7]. The putative function of the phosphoprotein P is to co-operate with the viral L protein to constitute the viral RNA-dependent RNA polymerase activity [8]. The functions of the other proteins encoded in the two mRNA species are not defined. Recently it has been suggested that the C and V proteins down-regulate viral transcription and replication respectively [9].

Following the suggestion that a capsid protein of adeno-associated virus [10] might use an ACG codon, it was directly shown that the Sendai virus C′ protein uses an ACG codon [4,5]. Since then, several viral and eukaryotic mRNAs have been shown to use non-AUG codons to initiate translation of regulatory amounts of the encoded proteins [11]. The mechanism by which a non-AUG start site is selected by the ribosome is not clear. However, it has been shown, for several proteins initiating at non-AUG start sites, that they begin their synthesis with a methionine residue [5,12]. These studies suggested that the initiation at a non-AUG start site occurs by a mechanism similar to that used for the AUG start site, which utilizes the ternary complex of eIF-2, $tRNA_f^{Met}$ and GTP. Moreover, it has been observed that mutants of the histidine coding frame in yeast that do not use an AUG start site can be recognized efficiently with mutants of eIF-2, further indicating the involvement of eIF-2 in the recognition process [13]. Curiously, initiation of c-*myc* protein 1 from a non-AUG codon (CUG) was stimulated 5–10-fold under conditions of methionine deprivation [14]. These results suggested that translation initiation can be regulated to enhance recognition of a suboptimal non-AUG codon. Recently we showed that not all mammalian mRNAs have the potential to utilize a non-AUG start site in mammalian cells. Our studies suggested that perhaps the P/C mRNA conformation is responsible for determining initiation at its non-AUG start site [7].

To investigate further the mechanism of initiation at the ACG start site of the C′ protein, we examined the effects of various modulators and inhibitors of protein synthesis on the P/C mRNA translation. We were surprised to observe that C′ synthesis, whether initiated from the native ACG start site or an alternative non-AUG (or an AUG) start site, was resistant to cycloheximide (CHX) and anisomycin, the inhibitors of peptidyl transferase. In fact, CHX stimulated C′ synthesis whereas other viral and cellular protein syntheses were significantly inhibited. In contrast, puromycin (a peptide chain terminator) and pactamycin (an inhibitor of peptide chain initiation) blocked synthesis of all the P/C mRNA encoded proteins with about equal efficiency. Hypotonic conditions also stimulated C′ synthesis in infected and transfected cells. These results suggest that conformational modulation of the mRNA is involved in the differential synthesis of the proteins encoded in the P/C mRNA. If this concept is correct, it opens a novel means of regulating protein expression by conformational changes in the mRNA species.

MATERIALS AND METHODS

Cell culture, virus infection and metabolic labelling

CV1 and COS1 cells were grown at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % (v/v) heat-

Abbreviations used: CHX, cycloheximide; DMEM, Hepes-buffered Dulbecco's modified Eagle's medium; pfu, plaque-forming unit; RIPA, radioimmunoprecipitation assay.

* To whom correspondence should be addressed.

† Present address: Laboratory of Animal Experiments, Institute of Immunological Science, Hokkaido University, Sapporo 060, Japan.

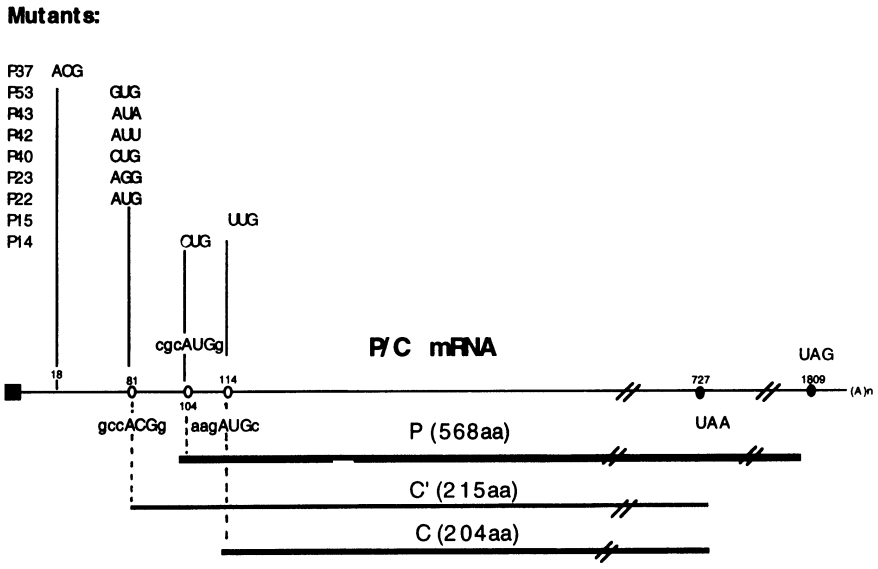


Figure 1 Schematic representation of the coding frames of the Sendai virus P/C mRNA

The positions (nucleotide number) of start sites of the C′, C and P proteins are presented with their sequence contexts. Termination sites of the P and C reading frames are also shown. Above each of the start sites, site-directed mutants used in the present study are shown, which are referred to by the numbers in the left column. A novel ACG start site created at nucleotide 18 for mutant P37 is shown. Abbreviation: aa, amino acids.

inactivated fetal calf serum. Subconfluent monolayers in 35 mm dishes were infected with 5 plaque-forming units (pfu) of Sendai virus (Z-strain) per cell and the infection was allowed to progress for 16–18 h. Before radiolabelling of proteins, medium was removed and the cells were incubated with 0.5 ml of methionine-free DMEM for 30 min with the indicated amounts (see the figures) of protein synthesis inhibitors. Cells were labelled with 50 μCi/ml Tran^{35}S-label (ICN) for 2–3 h and lysed with 0.5 ml of radioimmunoprecipitation assay (RIPA) buffer as described in detail previously [15].

cDNA constructs, site-directed mutants and transfections

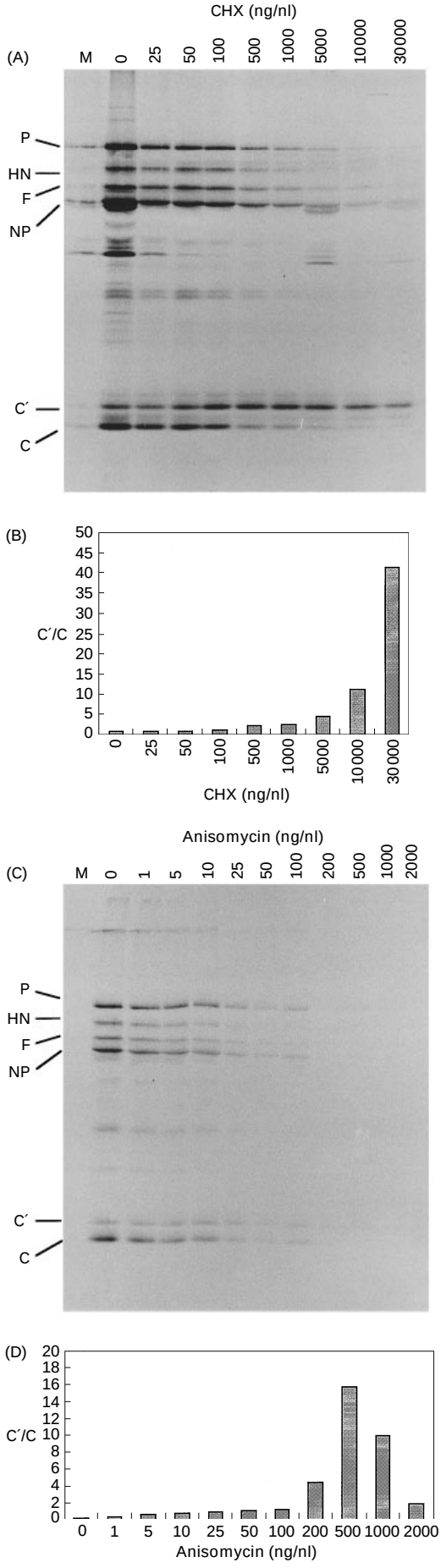
For transfections in COS1 cells, construct pBC/PC and its various mutants were used [6,15]. From these constructs, the P/C mRNA is expressed from the strong immediate-early promoter of cytomegalovirus [16]. Construction of site-directed mutants of the P/C mRNA has been described previously [17]. For transfections of CV1 cells, the P/C gene was subcloned behind the T7 promoter into pCDNAI/amp vector (Invitrogen) to obtain pcPC. Subconfluent (80–90 %) cell monolayers in 35 mm dishes were transfected with 3 μg of plasmid DNA by using Lipofectance (BRL) in accordance with the supplier's protocol. For expression in CV1 cells, the cells were infected with vaccinia virus (strain vTF7-3) at 5 pfu per cell, 30 min before transfection [18]. At 48 h after transfection of COS1 and at 24 h after transfection of CV1, cells were metabolically labelled and lysates prepared as described above.

Immunoprecipitation, gel electrophoresis and densitometric quantification

Polyclonal antiserum against Sendai virus proteins was raised in rabbits by using the lysed virions. This serum (1 μl) was mixed with antipeptide antiserum to C′ and C proteins (1 μl) [19] to immunoprecipitate the viral proteins from infected cell lysates (15 μl). Alternatively the P, C′ and C proteins were immunoprecipitated from infected (15 μl) or transfected (200 μl) cell lysates, using a mixture of monoclonal antibody to P (0.1 μl) [20] and anti-peptide antiserum to C′ and C proteins (1 μl). To precipitate the proteins quantitatively, antibodies were kept in excess of antigens. The immunocomplexes were adsorbed on 15 μl of Pansorbin (Calbiochem), pelleted and solubilized in Laemmli loading solution [21]. Proteins were resolved on SDS/12 % (w/v) polyacrylamide gels. Autoradiographs were scanned with a flat-bed scanner (ScanJet IIC, Hewlett Packard) and the protein bands were quantified densitometrically with the NIH Image software.

Preparation of RNA and Northern blot analysis

To analyse the expression of P/C mRNA from virus-infected COS1 cells, total cellular RNA from virus-infected cells was prepared by the guanidinium thiocyanate single-step procedure [22]. Briefly, cells were grown in 100 mm dishes to 80–90 % confluency and infected with Sendai virus at 5 pfu per cell. At 48 h after infection, cells were lysed with 4 M guanidinium thiocyanate in 42 mM sodium citrate/0.83 % *N*-laurylsarcosine/0.2 mM 2-mercaptoethanol. The lysate was immediately extracted with phenol/chloroform/isoamyl alcohol (25:24:1, by vol.). To determine the integrity and level of ribosomal RNA, 2 μg samples were analysed in 1 % (w/v) agarose gel containing 0.5 μg/ml of ethidium bromide. For Northern blot hybridization, 15 μg of each RNA sample was glyoxylated and resolved in 1.0 % agarose gel in 10 mM phosphate buffer, pH 7.0 [23]. RNA was capillary-transferred to Zeta-Probe (Bio-Rad) and hybridized with P/C mRNA-specific probe in accordance with the supplier's protocol. ^{32}P-labelled P/C gene-specific probe was prepared by using purified P/C gene as template with random heptameric oligonucleotide primers and [α-^{32}P]dCTP [24]. After hybridization the blot was exposed to X-ray film for 4–24 h.



RESULTS

Effects of protein synthesis inhibitors

Our cell-free translation experiments indicated that the synthesis of the various proteins encoded in the P/C mRNA is differentially affected by protein synthesis inhibitors. Owing to the inherent variability of the cell-free systems and the high sensitivity of P/C mRNA translation to Mg^{2+}, Na^{+}, K^{+} and spermidine ([25]; K. C. Gupta, unpublished work), we examined the effects of various protein synthesis inhibitors in Sendai virus-infected or in the P/C gene-transfected cells. Virus-infected cells were exposed to the indicated concentrations of an inhibitor for 30 min and the proteins were labelled with Tran^{35}S-label (75% methionine/15% cysteine; ICN) for 2 h in the presence of the inhibitor. Among the various inhibitors tested, CHX, an inhibitor of peptide chain elongation, had a surprising effect on the translation of the P/C mRNA. As expected, CHX inhibited the synthesis of P, C and other viral proteins in a concentration-dependent manner (Figure 2A). At 30 μg/ml CHX, synthesis of P and C decreased by more than 95%. Similarly, at this concentration of CHX, synthesis of most of the cellular and other viral proteins (NP, F and HN) was either significantly decreased or entirely abolished. In contrast, CHX (up to 30 μg/ml) stimulated C′ synthesis. Consequently the ratio of C′/C increased progressively from 0.4 in the absence of CHX to 40 at 30 μg/ml CHX (Figure 2B). However, higher concentrations of CHX (more than 30 μg/ml) inhibited C′ synthesis in a dose-dependent manner (results not shown). Another potent inhibitor of peptide chain elongation, anisomycin, which interferes with a step earlier in the peptidyl transferase reaction than CHX [26], similarly blocked viral and cellular protein synthesis. Although the synthesis of C′ was resistant to the inhibitory effect of anisomycin (Figure 2C), it did not exhibit the strong stimulatory effect of CHX. Anisomycin inhibited all cellular and viral protein synthesis at concentrations as low as 1 ng/ml. However, the synthesis of C′ was not inhibited up to 0.1 μg/ml; its synthesis then continued at approx. 25% of maximum at 1 μg/ml anisomycin (Figures 2C and 2D). In fact, C′ synthesis was slightly stimulated (10–20%) at 5–10 ng/ml anisomycin. As with the CHX effect, the ratio of C′/C increased from 0.3 in the absence of anisomycin to 15.7 at 0.5 μg/ml anisomycin (Figure 2D). Thus C′ synthesis was resistant to both inhibitors of peptide chain elongation. Although C′ synthesis was significantly stimulated by CHX, it was resistant to inhibition by anisomycin.

To determine whether other protein synthesis inhibitors such as puromycin (a peptide chain terminator) and pactamycin (an inhibitor of peptide chain initiation) affected P/C mRNA translation, we examined their effects in virus-infected cells. Both these inhibitors suppressed all protein synthesis, including that of the C′ protein, in a dose-dependent manner. Puromycin (50 μg/ml) decreased P and C synthesis to 20%, whereas C′ synthesis was not detected (Figure 3, upper panel). Interestingly, puromycin at lower concentrations (1–5 μg/ml) stimulated (10–20%) overall protein synthesis. At 400 ng/ml, pactamycin entirely blocked cellular and viral protein synthesis (Figure 3, lower

Figure 2 Effect of inhibitors of peptidyl transfer reactions on the synthesis of Sendai virus proteins

(**A**) Effects of increasing concentrations (0–30 μg/ml) of CHX; (**B**) ratios of C′/C at these concentrations of CHX; (**C**) effect of increasing concentrations (0–2 μg/ml) of anisomycin; (**D**) ratios of C′/C. Sendai virus proteins were immunoprecipitated from cell lysates (15 μl) by using a mixture of rabbit antisera directed against lysed Sendai virions and to peptides corresponding to the C′ and C proteins, as described in the Materials and methods section, and resolved in 12% Laemmli gels.

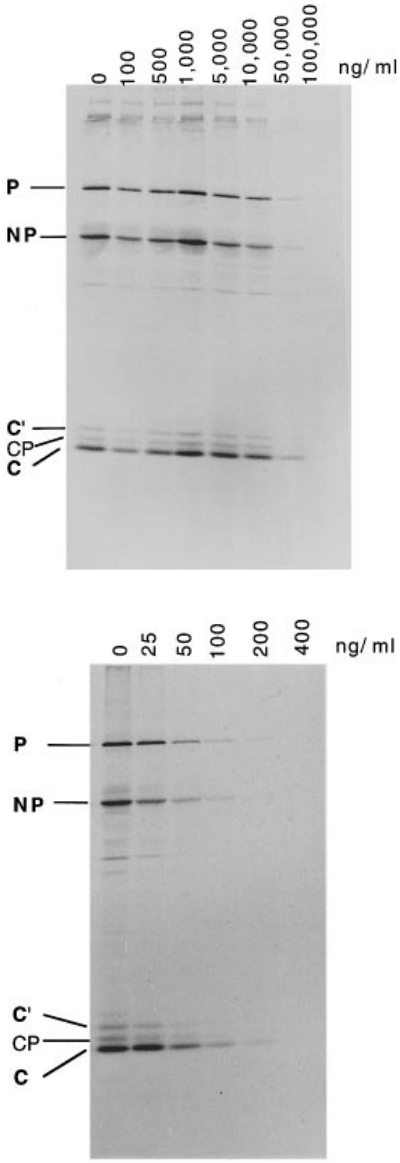


Figure 3 Effect of a peptide chain terminator (puromycin) and an inhibitor of chain initiation (pactamycin) on translation of the P/C mRNA encoded proteins in Sendai virus-infected cells

Effect of increasing concentrations of puromycin (upper panel) or pactamycin (lower panel). The P, C′ and C proteins were immunoprecipitated from virus-infected cell lysates (15 μl) by using a mixture of anti-P monoclonal antibodies and anti-C peptide antibodies as described in the Methods and materials section. Because P protein binds to NP protein, NP protein co-precipitates with the P protein. A protein band present between C′ and C is the phosphorylated form of the C protein; it was designated CP [19].

panel). These results showed that CHX, anisomycin, puromycin and pactamycin inhibited protein synthesis in an expected dose-dependent manner, except that C′ synthesis was not affected in the presence of CHX and anisomycin. These results also show that the C′ protein is synthesized *de novo* in virus-infected cells and that CHX is not increasing the stability of the C′ protein. It is therefore possible that C′ protein synthesis occurs via a mechanism that is not affected by low levels of inhibitors of chain elongation.

To determine whether the P/C mRNA was transported by some mechanism into mitochondria, where the C′ protein was translated by the CHX-insensitive ribosomes of mitochondria, we treated Sendai virus-infected cells with chloramphenicol. It is known that mitochondrial protein synthesis, unlike cytoplasmic protein synthesis, is inhibited by chloramphenicol [26]. Chloramphenicol did not inhibit C′ synthesis or the synthesis of other P/C mRNA-encoded proteins (results not shown). These results ruled out the possibility that the C′ protein was synthesized by CHX-insensitive ribosomes in mitochondria. Moreover, these results indicated that C′ is synthesized in the cytoplasm.

Other Sendai virus proteins and virus infection are not necessary for the exceptional effect of CHX

The functional significance of the C′ protein in virus-infected cells is unknown. However, it is possible that other viral proteins could interact with C′ or the P/C mRNA in allowing preferential C′ synthesis in the presence of CHX. To determine whether the stimulation of C′ synthesis by CHX was due to its effect on other Sendai virus proteins, we expressed the P/C gene in CV1 cells. The P/C cDNA was transfected under the control of T7 promoter (recombinant pcPC) in CV1 cells infected with the recombinant vaccinia virus vTF7-3. This system allows the expression of P/C mRNA in the cytoplasm, where the P/C mRNA is synthesized in Sendai virus-infected cells. Essentially identical effects were observed on C′ synthesis in response to CHX as were detected in Sendai virus-infected CV1 cells (Figure 4, upper panel). However, infection with vaccinia virus could cause changes in the cellular milieu and alter P/C mRNA translation in transfected cells. To test this possibility, P/C cDNA under the control of CMV immediate-early promoter (pBC construct) was transfected in COS-1. With this recombinant, P/C mRNA is expressed in the nucleus and is transported to the cytoplasm. This is in contrast with the cytoplasmic expression of P/C mRNA in Sendai virus-infected cells. Similar results were observed in transfected cells in response to CHX (Figure 4, lower panel), indicating that virus infection itself was not involved in the stimulation of C′ synthesis by CHX. Moreover, when the P/C mRNA is expressed in the nucleus with pBCPC, the mature mRNA is synthesized from a precursor RNA species. The precursor RNA is processed at the 5′ and 3′ termini to yield an additional 75 nucleotides at the 5′ end and approx. 350 nucleotides at the 3′ terminus respectively [27]. Apparently the additional terminal sequences in the P/C mRNA had no influence on the stimulation by CHX. These results suggested that stimulation of C′ synthesis by CHX is an inherent characteristic of the Sendai virus P/C mRNA irrespective of whether the mRNA is synthesized in the cytoplasm or in the nucleus.

C′ synthesis is unusual irrespective of non-AUG start site

As discussed above, we showed that the synthesis of C′ is initiated at an ACG codon [5]. It is possible that the stimulation of C′ synthesis by CHX is due to its non-traditional start site. To test this possibility, we used alternative start codons in place of ACG. Our previous studies have shown that CUG (in mutant P40) and GUG (in mutant P53) codons are recognized as the start sites for the C′ protein by ribosomes with a greater efficiency than the ACG start site (Figure 1) [15]. To determine whether the synthesis of C′ initiated at an alternative non-AUG start site would be similarly stimulated, we transfected the mutant P/C gene in COS-1 cells and examined the effect of CHX at 1 μg/ml. Results described in the previous section showed that 1 μg/ml CHX gave the maximum stimulation of the C′ synthesis in virus-infected cells (see Figures 2A and 4). We also examined the effect of CHX on a mutant (P37) in which an additional ACG codon (at nucleotide 18) was created 63 nucleo-

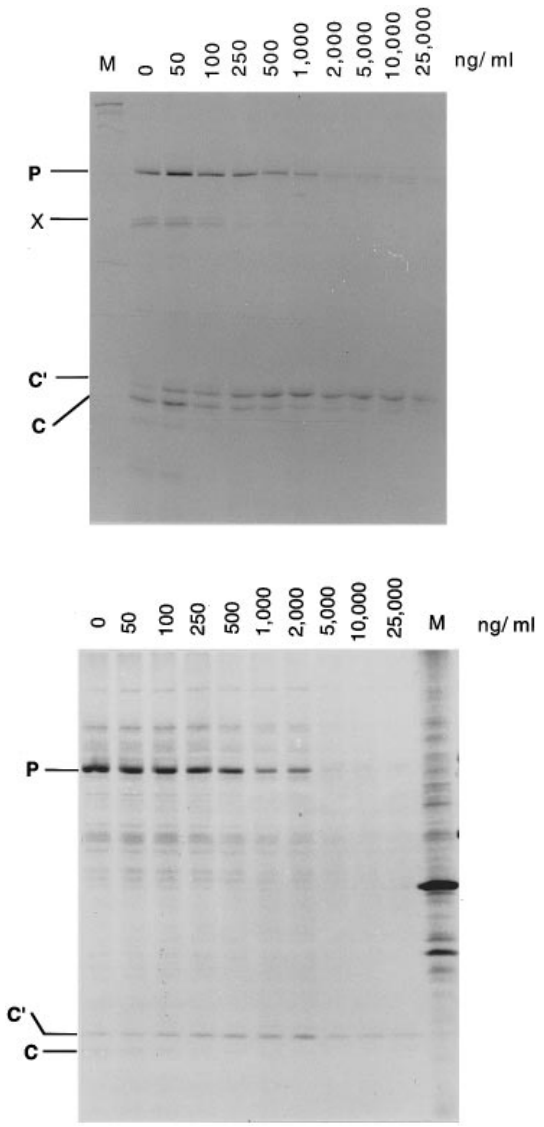


Figure 4 Effect of increasing concentrations (0–25 μg/ml) of CHX on P/C mRNA translation in P/C gene-transfected cells

Upper panel: plasmid pcPC was transfected into CV1 cells infected with vaccinia virus vTF7-3. The transfected cells were treated for 30 min with the indicated concentrations of CHX, labelled with Tran^{35}S-label; P, C′ and C were analysed as described for Figure 2. X denotes a non-specific protein band that co-immunoprecipitates with the P protein from vaccinia virus-infected cell lysates. Lower panel: plasmid pBC/PC was transfected into COS1 cells. At 48 h after transfection, cells were treated with indicated concentrations of CHX for 30 min, labelled and analysed as described in the legend to Figure 2. M represents mock-transfected and labelled COS1 cell lysate.

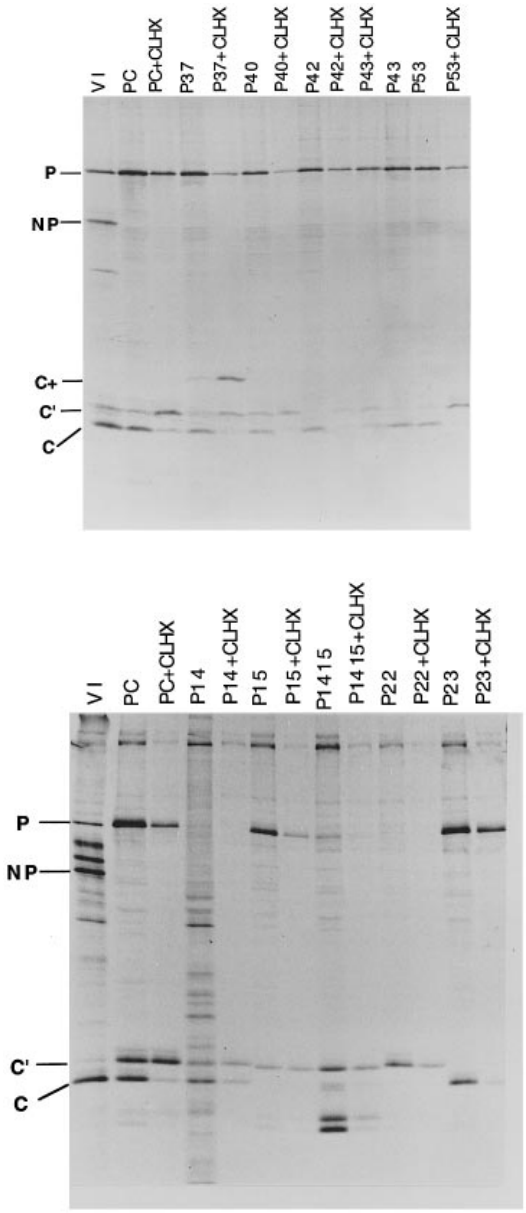


Figure 5 Effect of CHX (1 μg/ml) on the various start site mutants (see Figure 1) of the P/C mRNA

Mutant plasmids of the P/C gene were transfected in COS-1 cells. The cells were treated with 1 μg/ml CHX for 30 min and labelled; P, C′ and C proteins were analysed as described in the legend to Figure 2. Parallel lanes show the expression of each mutant in the presence or absence of CHX (CLHX). Upper panel: various non-AUG start site mutants of the P/C mRNA. The position of the novel C+ protein initiating from an ACG codon created at nucleotide 18 (mutant P37) is shown. Lower panel: mutants of AUG start sites of the P and C proteins (mutants P14, P15 and P1415) and AUG mutant (P22) and non-start mutant (P23) of the C′-ACG start site. For some reason, C′ synthesis in this batch of experiments was stimulated in the absence of CHX (compare the PC lane in the upper panel with that in the lower panel). Therefore the lower panel shows a resistance of C′ synthesis to CHX rather than stimulation. The two proteins smaller than the C protein overexpressed in P1415 are Y1 and Y2 [6].

tides upstream of the C′ start site in the C-reading frame (Figure 1). We have shown previously that this upstream start site was used as efficiently as the native ACG to synthesize an N-terminally extended C protein (C+) [15]. Interestingly, except for the CUG mutant (P40), C′ synthesis from all other non-AUG start codons and C+ synthesis from the upstream ACG start site was stimulated by CHX (Figure 5, upper panel). Although no stimulation of C′ synthesis was noticed in P40, its synthesis remained resistant to CHX. In the mutant P37, not only C+ but also C′ synthesis was stimulated. The synthesis of C′, which was barely detectable owing to inefficient initiations from AUU (mutant P43) and AUA (mutant P42) codons [15], became clearly visible under stimulation by CHX (Figure 5, upper panel). We have shown previously that the various non-AUG codons were used with different efficiencies to initiate C′ synthesis [15]. However, in the presence of CHX the level of C′

synthesis is very similar. These results showed that the synthesis of C′ protein was unusual whether the initiation was from the native ACG start site or from an alternative non-AUG start site. These results also showed that the stimulation of C′ synthesis by CHX was not dependent on the position of the start site as evidenced by C+ synthesis. These results, consistent with our previous report [7], suggested that the local RNA structure at the C′ start site is not important for the CHX-resistant mode of C′ synthesis.

Next we tested the possibility that C′ synthesis itself was unusual irrespective of the nature of the start site. To test this possibility we used a mutant (P22) in which the C′ ACG start site was mutated to AUG [15]. This mutation created an optimum AUG upstream of all the translation start sites in the P/C mRNA. Consistent with the ribosomal scanning model [28], creation of an AUG codon upstream of all the other start sites in the P/C mRNA blocked the synthesis of other P/C mRNA-encoded proteins (Figure 5, lower panel). Although CHX (1 μg/ml) did not stimulate the synthesis of the C′ protein from the AUG start site, synthesis of the C′ protein was decreased to only 50% (Figure 5, lower panel). This remaining level of C′ synthesis is similar to that observed in the presence of CHX in various non-AUG mutants (see Figure 5, upper panel). Curiously, in the presence of 50 μg/ml CHX, at which level all viral and cellular protein synthesis is abolished, the level of C′ synthesis from P22 corresponded well with that of C′ expressed from the wild-type P/C gene (results not shown). Interestingly, these results showed that only 50% of the C′ protein synthesized from P22 is resistant to CHX. These results confirm that C′ does not have to initiate at a non-AUG start site to be resistant to CHX. Therefore to test whether a non-start codon would be used for the synthesis of the C′ protein in the presence of CHX, we used mutant P23, in which ACG was changed to AGG (Figure 1) [15]. No initiation was observed at this codon in the presence of CHX (Figure 5, lower panel). These results showed that a translation start site, whether AUG or non-AUG, is necessary for initiation, stimulation and/or resistance of C′ synthesis to CHX.

Other start sites of the P/C mRNA have no relation to the stimulation of C′ synthesis by CHX

Downstream of the C′ start site are the start sites for the P and C proteins (Figure 1). It is possible that the inhibition of elongation of P and C proteins by CHX stimulates C′ synthesis. To determine whether synthesis of the P and C proteins is somehow involved in the CHX stimulation, we mutagenized the P-AUG and C-AUG start sites to CUG (mutant P14) and UUG (mutant P15) respectively. Finally both mutants were combined into one mutant P1415. These mutants were expressed in COS-1 cells as described above. Irrespective of the presence or absence of the P or C AUG start sites, C′ synthesis was resistant to CHX (Figure 5, lower panel). These results clearly showed that the peptide chain initiation/elongation at the downstream start sites is not involved in the stimulation of C′ synthesis. Here again the level of C′ protein in the presence of CHX is similar to that observed for other mutants. Moreover, these results indicated that the P and C proteins could not be the cause of CHX-resistant C′ synthesis.

Taken together, the results suggest that the mode of C′ protein synthesis is different from the synthesis of other viral and cellular proteins. This is possible if C′ were to interact with initiation factors to stimulate its own initiation or to interact with ribosomes or elongation factors to counteract their inhibition by CHX. However, this model would not explain the highly specific stimulation of C′ protein synthesis alone in the face of strong inhibition of the synthesis of P and C proteins encoded in the same mRNA. Thus an alternative mechanism must exist for the preferential synthesis of C′ from the P/C mRNA.

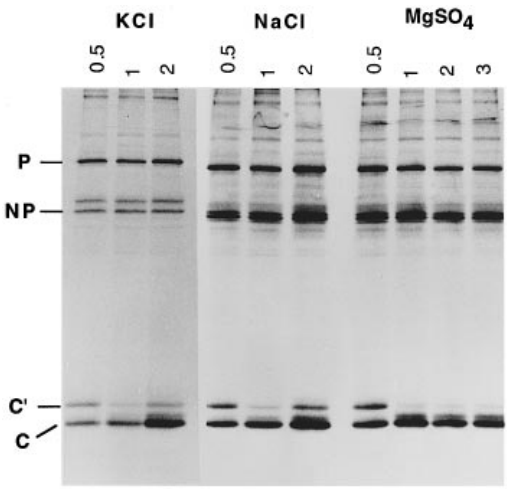


Figure 6 Effect of hypotonic and hypertonic growth media on the expression of P/C mRNA-encoded proteins in virus-infected cells

Sendai virus-infected CV1 cells were exposed for 16 h to half-strength (0.5) medium, or to the medium containing double (2) the regular DMEM concentration of KCl (800 mg/l) or NaCl (12.8 μg/l), or double (2, 195 mg/l) or triple (3, 293 mg/l) the concentration of DMEM $MgSO_4$. Proteins were labelled in corresponding methionine-deficient media. The P, C′ and C proteins were immunoprecipitated as described in the Materials and methods section, and proteins were resolved in 12% Laemmli gels.

Stimulation of C′ synthesis by hypotonic conditions

An alternative possibility is that the P/C mRNA exists intracellularly in two different conformations, conformation B allowing synthesis of the C′ protein, and conformation A allowing synthesis of the P and C proteins. In the presence of CHX and associated translation components, conformation B is stabilized and conformation A is destabilized. Consequently all or most of the P/C mRNA is in conformation B, which allows C′ synthesis. As the P/C mRNA is translated quite efficiently, most probably because of high ribosome loading [15], this conformational shift might cause a greater amount of C′ protein synthesis. To test the possibility that the mRNA conformation is modulating the expression of the proteins encoded in the P/C mRNA, we exposed the virus-infected cells to hypotonic and hypertonic media. It was interesting to note that under hypotonic conditions (half the concentration of the growth medium) C′ synthesis increased by approx. 5-fold compared with that in the isotonic condition (Figure 6). However, hypertonic conditions (normal medium plus additional NaCl or KCl) stimulated synthesis of all the P/C mRNA-encoded proteins. These results suggest that the preferential stimulation of C′ synthesis in hypotonic conditions could be due to conformational changes in the P/C mRNA.

CHX has no effect on P/C mRNA synthesis

One possibility is that CHX alters P/C mRNA synthesis such that an alternate subgenomic mRNA is synthesized that allows the synthesis of the C′ protein only. To test this possibility, total cellular RNA was prepared from Sendai virus-infected cells after 4 h of treatment with CHX (1 μg/ml). RNA from CHX-treated

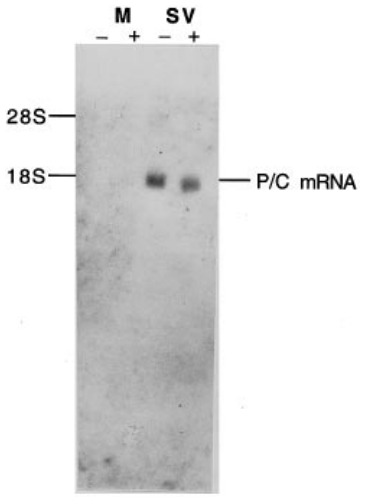


Figure 7 Northern blot hybridization of the P/C mRNA from Sendai virus-infected cells

Total cellular RNA (15 μg each) from mock-infected (M), Sendai virus-infected (SV), CHX (1 μg/ml)-treated (+) and -untreated (−) cells were glyoxylated and resolved in a 1% (w/v) agarose gel. After transfer to the Zeta-Probe (Bio-Rad) membrane, the blot was hybridized with a P/C mRNA-specific probe. The gel migration positions of the glyoxylated 18 S and 28 S rRNA species are indicated. P/C mRNA was detected only in SV-infected cells.

and non-treated cells were analysed by Northern blot and hybridized with the P/C mRNA specific probe. Both RNA samples showed the presence of the same level of P/C mRNA identical in size (Figure 7). Even a very long exposure of the blot to X-ray film did not reveal any difference in the specific profiles of P/C mRNA in the two samples. These results lead us to conclude that the observed stimulation of C′ synthesis by CHX is due to a modulation of protein synthesis and not of the mRNA synthesis.

DISCUSSION

It is well established that CHX is a potent inhibitor of eukaryotic protein synthesis. It blocks the peptidyl transfer reaction during chain elongation [29]. Resistance to CHX in yeast and *Tetrahymena* could be acquired by mutations in one of the three ribosomal proteins L21, L29 and L41 [30–33]. All three proteins are components of the large subunit of the ribosome that is primarily involved in peptide chain elongation. Mutation of any of these proteins confers CHX resistance on the entire protein synthesis machinery. It was therefore rather surprising to find that synthesis of the Sendai virus C′ protein alone was resistant to CHX. It has previously been observed that CHX (0.1 μg/ml) induced the synthesis of three distinct heat-shock-related proteins in *Neurospora* [34]. Similarly, the translation of a certain class of mouse fibroblast mRNA species was stimulated by CHX [35]. Normally these mRNA species were poorly translated and thus were present in small polyribosomes; however, they were driven to large polyribosomes by CHX. Thus repressors might exist to limit translation of these mRNAs. However, the stimulation of C′ synthesis and the inhibition of P and C synthesis from the same mRNA cannot be resolved by polyribosome profile shift.

The stimulation or the inhibition of translation of specific mRNA species, for instance those of stress- and metabolite-regulated proteins such as GCN4, ornithine decarboxylase, ferritin and heat shock, has been observed depending on the cellular milieu or external effectors [36]. Similarly, initiation from a CUG start site in the c-*myc* mRNA was favoured over an AUG start site under conditions of methionine deprivation [14]. However, we showed that the stimulation of C′ synthesis by CHX is not due to the initiation of synthesis at a non-traditional start site, that is, an ACG codon. Surprisingly, the level of C′ synthesis in the presence of CHX in various P/C mutants is very similar. These results suggest that in the presence of CHX the nature of the start codon does not influence the initiation efficiency of the C′ protein. Although a start site is necessary for C′ synthesis, other undetermined factors play a role in its level of synthesis. As far as we know, this is the first example of an mRNA in which simultaneous stimulation and inhibition of protein synthesis have been observed.

Previously we suggested that the initiation of C′ synthesis is regulated by a higher-order structure of the P/C mRNA [7]. Recently we showed that there is no unusual secondary structure in the 5′ proximal 200 nucleotides of the P/C mRNA [6]. However, these results did not rule out the possibility of higher-order structures created by long-distance interactions. The results in this paper are consistent with the concept that higher-order structure of the P/C mRNA regulates translation of this mRNA. The apparently contradictory effects of CHX on P/C mRNA translation could be explained if the mRNA were to exist in alternative conformations. Depending on the relative abundance of the alternative conformations, one protein or other could be preferentially synthesized. On the basis of the results presented here and elsewhere, we hypothesize that the Sendai virus P/C mRNA exists in at least two alternative conformations: A and B. Under natural conditions, whereas the abundant A conformation allows efficient synthesis of the P and C proteins, the alternative, less abundant, B conformation allows C′ synthesis. However, the relative ratio of the two conformations could alter depending on the conditions of cell growth. Stimulation of C′ synthesis under hypotonic conditions supports this concept. Similarly, the modulatory effects of Mg^{2+}, Na^{+}, K^{+} and spermidine on cell-free synthesis of C′, C and P proteins further favour this concept ([25], and K. C. Gupta, unpublished work). Although there is no information suggesting that CHX modulates the conformation of mRNA, our results lead us to hypothesize that CHX is most probably causing a shift in the natural ratio of alternative conformations of the P/C mRNA. Nevertheless it will be very important to demonstrate directly that alternative conformations are indeed involved in regulating the synthesis of various proteins from the P/C mRNA. Currently no methods are available that could examine the conformations of large RNA molecules (approx. 2000 nucleotides). If the concept of alternative conformations is correct, it raises the possibility that the expression of other mRNA species, such as those of ornithine decarboxylase, heat shock and c-*myc*, could also be regulated by alteration in their conformations. This regulation could be brought about quite rapidly in response to changes in the external or internal milieu of cells. This would be a novel and additional mechanism for regulating gene expression at short notice.

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