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Characterization of the Thermosensitive ts453 Reovirus Mutant: Increased dsRNA Binding of σ3 Protein Correlates with Interferon Resistance

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The mutation harbored by the reovirus ts453 thermosensitive mutant has been assigned to the S4 gene encoding the major outer capsid protein σ3. Previous gene sequencing has identified a nonconservative amino acid substitution located near the zinc finger of σ3 protein in the mutant. Coexpression in COS cells of the σ3 protein presenting this amino acid substitution (N16K), together with the other major capsid protein μ1, has also revealed an altered interaction between the two proteins; this altered interaction prevents the σ3-dependent cleavage of μ1 to μ1C. This could explain the lack of outer capsid assembly observed during ts453 virus infection at nonpermissive temperature. In the present study, we pursued the characterization of this mutant σ3 protein. Although the N16K mutation is located close to the zinc finger region, it did not affect the ability of the protein to bind zinc. In contrast, this mutation, as well as mutations within the zinc finger motif itself, can increase the binding of the protein to double-stranded RNA (dsRNA). It also appears that the N16K mutant protein is more efficiently transported to the nucleus than the wild-type protein, an observation consistent with the postulated role of dsRNA binding in σ3 nuclear presence. The lack of association with μ1, and/or the increased dsRNA-binding activity of σ3, could be responsible for a partial resistance of the ts453 virus to interferon treatment and this could have important consequences in the context of protein synthesis regulation during natural reovirus infection.

INTRODUCTION

The genome of mammalian reoviruses consists of 10 double-stranded RNA segments enclosed in an inner and an outer protein shell (reviewed by Nibert *et al.,* 1996). Each of these RNA segments encodes a viral protein, with the exception of the dicistronic S1 gene, which produces two proteins encoded in different reading frames. The nature of the viral genome and its mode of replication has precluded the development of a reverse-genetic strategy for the study of reovirus protein functions. However, the segmented nature of the genome has allowed the generation of reassortant viruses and facilitated the assignment of temperature-sensitive (ts) mutations to specific genes (Ramig and Fields, 1983; Nibert *et al.,* 1996). Hence, reovirus thermosensitive mutants represent an interesting alternative approach to study the functional roles of viral proteins. Such a conditional temperature-sensitive mutant is the ts453 virus derived from reovirus serotype 3 Dearing (T3D) and belonging to complementation group G (Cross and Fields, 1972); the mutation was assigned to the S4 gene, known to encode the σ3 viral protein (Mustoe *et al.,* 1978).

The σ3 protein is present in about 600 molecules per mature virion and, along with μ1C, is one of the two major components of the outer capsid (Metcalf *et al.,* 1991; Nibert *et al.,* 1996). Its association with μ1 contributes to the stability of μ1, thus allowing its cleavage to μ1C (Tillotson and Shatkin, 1992; Mabrouk and Lemay, 1994b). In addition to its structural role, σ3 harbors two functional domains that can be separated following V8 protease digestion: an amino-terminal domain that has a zinc-binding motif and a carboxy-terminal region that has affinity for double-stranded RNA (dsRNA) (Schiff *et al.,* 1988; Miller and Samuel, 1992; Wang *et al.,* 1996).

The amino-terminal domain of σ3 appears to be involved in the interaction with μ1, as first suggested by the study of the ts453 thermosensitive mutant of the virus. The mutant is defective in outer capsid assembly when grown at nonpermissive temperature and exhibits a nonconservative mutation near the zinc-binding motif of σ3 (Danis *et al.,* 1992). It appears that misfolding of the thermosensitive σ3 protein expressed at the nonpermissive temperature could be involved in phenotypic changes associated with this mutation (Shing and Coombs, 1996). When coexpressed in COS cells, the σ3 protein presenting this unique amino acid substitution fails to interact with μ1, thereby preventing μ1C accumulation (Mabrouk and Lemay, 1994b). Additional studies using either cotransfection of μ1 and σ3 expression vectors or *in vitro* translated proteins further support the importance of σ3 amino-terminal domain in its interac-

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tion with μ1 (Mabrouk and Lemay, 1994b; Shepard *et al.,* 1996).

As for the dsRNA-binding activity of σ3, it is believed to be involved in translational regulation through an inhibition of the cellular double-stranded RNA-dependent protein kinase (PKR) (Giantini and Shatkin, 1989; Lloyd and Shatkin 1992; Seliger *et al.,* 1992; Martin and McCrae, 1993; Beattie *et al.,* 1995; Mabrouk *et al.,* 1995). Viral infection and treatment of cells with interferon are known to induce a higher PKR expression level, the protein acting as a host–cell antiviral mechanism; accordingly, numerous viruses have evolved strategies to counteract PKR action (reviewed by: Samuel, 1991; Katze, 1992, 1993; Hovanessian, 1993). In addition to its anti-PKR action, the affinity of σ3 for double-stranded RNA appears important for the partial nuclear localization of the protein (Yue and Shatkin, 1996).

The property of σ3 to bind dsRNA is conferred by the presence of two basic motifs, within a portion of the ~85-amino-acid carboxy-terminal domain (Miller and Samuel, 1992; Denzler and Jacobs, 1994; Mabrouk *et al.,* 1995; Wang *et al.,* 1996). Northwestern blot analyses of full-length and amino-truncated proteins have revealed that the second basic motif is more important for RNA–protein interaction (Miller and Samuel, 1992; Wang *et al.,* 1996); in the absence of the N-terminal portion of the protein, mutations affecting the first basic motif appeared to have only minimal effect on RNA binding (Wang *et al.,* 1996). Northwestern blot has not only revealed that the zinc finger is dispensable for dsRNA binding, but also suggested that the amino-terminal portion of σ3 could exert an inhibitory effect on the dsRNA affinity conferred by the carboxy-terminal portion of the protein (Schiff *et al.,* 1988; Miller and Samuel, 1992; Wang *et al.,* 1996).

In the present study the thermosensitive mutant protein was found to exhibit an increased dsRNA binding and an altered subcellular distribution. The effect of these properties on interferon sensitivity was thus investigated. Mouse L929 cells treated with interferon were infected with either virions or ISVPs (infectious subviral particles); the synthesis of viral proteins in ts453-infected cells was found to be resistant to interferon when compared to the sensitivity in cells infected with the wild-type virus. This further supports the idea that the dsRNA-binding activity of σ3 can act as a major determinant in reovirus resistance to interferon. The difference in interferon sensitivity between wild-type and ts453 virus was also found to be mostly abolished when infections were carried out with infectious subviral particles.

RESULTS

Effects of amino-terminal mutations on dsRNA binding

In an effort to elucidate the effects of the amino-terminal portion of σ3 on dsRNA binding, pET expression vectors were first designed to harbor mutations within the region encoding the N-terminal domain of the protein. The amino acid substitutions used were the ones already described as affecting zinc binding (C51A and C73A) (Mabrouk *et al.,* 1994a) and the nonconservative substitution (N16K) previously found in the thermosensitive ts453 virus (Danis *et al.,* 1992). In turn, these substitutions were combined to mutations affecting the first C-terminal basic motif of the protein (K,R234–240A or R236A basic amino acid substitutions) (Wang *et al.,* 1996). The positions of the different amino acid substitutions are summarized in Fig. 1. The different proteins were expressed in bacterial cells by taking advantage of the T7 promoter/polymerase system (Studier *et al.,* 1990). The capacity of each mutant to bind dsRNA was analyzed by performing a Northwestern blot assay on full-length proteins. The amino-terminal amino acid substitutions (C51A, C73A, or N16K) did increase σ3 ability to bind dsRNA by two- to four-fold compared to the wild-type protein (Figs. 2A and 2C); the presence of identical amounts of filter-bound proteins was verified by fast green staining (Fig. 2B). Moreover, the mutations affecting the first C-terminal motif, known to reduce RNA binding, had less effect when combined to the N-terminal amino acid substitutions. In fact, it was possible to recover RNA binding to an even higher than wild-type level by associating the R236A substitution with either one of the mutations affecting the amino-terminal region of the protein (Fig. 2A, lanes 6, 9, and 12, and Fig. 2C). However, only limited recovery was observed when the more drastic substitution of four basic amino acids was introduced in the same C-terminal motif (Figs. 2A, lanes 5, 8, and 11, and 2C).

Two additional results also indicate an increased affinity of N16K for dsRNA. Affinity chromatography on a synthetic dsRNA column, using σ3 proteins produced in COS cells, has shown that the binding of the σ3 mutant is more resistant to an increase in ionic strength from 0.125 to 0.4 M (Fig. 3A). Also, the maximal activity of the N16K protein in the COS cell cotransfection assay, using stimulation of expression on a reporter CAT gene, was reached at lower amounts of cotransfected σ3 expression vector (Fig. 3B). This assay, in which σ3 exerts a translational effect via PKR inhibition, was previously shown to be related to the ability of σ3 to bind dsRNA (Mabrouk *et al.,* 1995; Yue and Shatkin, 1997).

Effect of the thermosensitive mutation on zinc binding

Initially, the ts453 mutant was shown to be defective in outer capsid assembly (Morgan and Zweerink, 1974; Danis *et al.,* 1992). In addition to preventing σ3–μ1 interaction (Mabrouk and Lemay, 1994b), the preceding experiments showed that the N16K substitution increases dsRNA binding. Since the N16K substitution is located

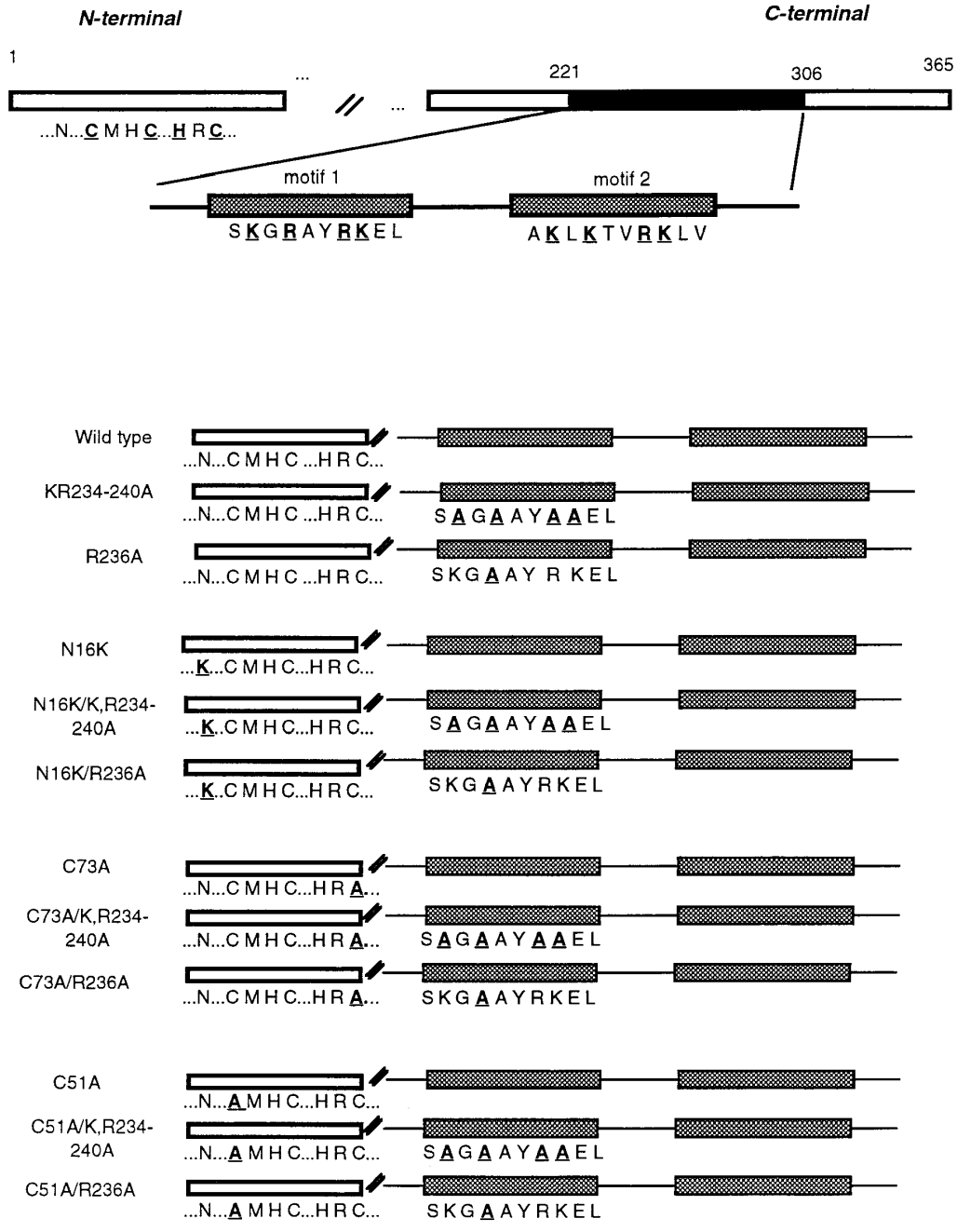

FIG. 1 Schematic representation of the different mutations within the N-terminal portion and the first C-terminal basic motif of σ3 expression vectors. Underlined bold amino acids constitute the four amino acids responsible for the tetracoordination of zinc atom and the basic amino acids of the two C-terminal motifs, previously shown to be involved in dsRNA binding. The mutations used in this study are also indicated as bold underlined letters.

near the zinc finger and since it has been recently reported that alterations of the zinc-binding motif can also preclude σ3–μ1 association (Shepard *et al.,* 1996), it should be determined whether the N16K substitution directly interferes with the ability of the protein to bind zinc. This point was examined by an assay based on a modification of zinc-chelate affinity chromatography using radiolabeled proteins produced from transfected COS-7 cells. In this assay, proteins retained on the column through their interaction with zinc ions are eluted with a chelating agent (EDTA) and analyzed by SDS–PAGE and autoradiography. A similar percentage of σ3 wild-type (13%) or N16K protein (19%) was retained onto the column (Fig. 4B, lanes 2 and 3). In contrast, amino acid substitutions introduced directly into the zinc finger prevented binding even when larger amounts of the protein were applied on the column (Mabrouk and Lemay, 1994a; Fig. 4B, lane 4, and data not shown).

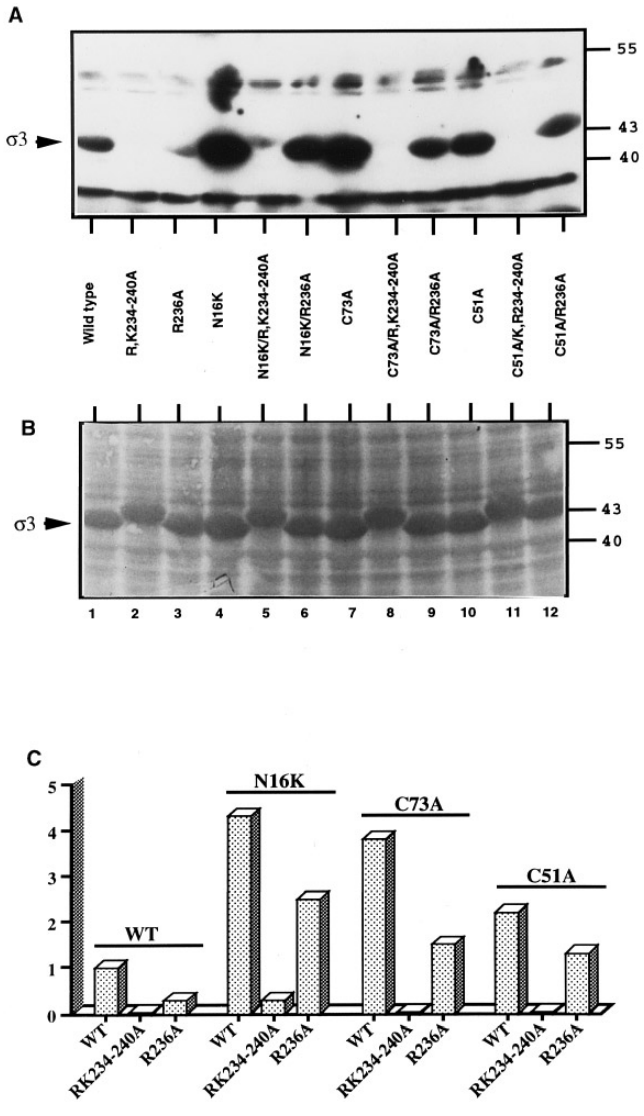

FIG. 2. Northwestern blot analysis of double-stranded RNA binding in different σ3 mutant proteins. The σ3 mutants were expressed in bacterial cells following IPTG induction and bacterial pellets were analyzed by SDS–PAGE (see Materials and Methods). A is the autoradiogram of the Northwestern analysis using radiolabeled reovirus dsRNA. B is the result of fast green staining of the proteins transferred onto the filter, indicating similar expression levels of the different mutants used in the assay. The position of the σ3 proteins is indicated. As previously noticed, proteins harboring the K,R234–240 mutation exhibit a reduced electrophoretic mobility. C shows quantitation of dsRNA binding relative to the wild-type value.

Subcellular localization of the thermosensitive mutant σ3 protein

Although mechanistic details are still lacking, recent results suggest that σ3 is partly transported to the nucleus by means of its dsRNA-binding activity (Yue and Shatkin, 1996). The thermosensitive σ3 mutant having an increased affinity for dsRNA, it was thus of interest to determine whether this property could actually influence the subcellular localization of the protein. COS-7 cells were transfected with either wild-type or N16K expression vectors. Cell fractionation showed nearly equal amounts of wild-type σ3 in all three subcellular fractions (cytoplasmic, cytoskeletal, and nuclear) (Fig. 5, lanes 2–4), whereas a larger proportion of the mutant protein was found in the nuclear fraction, 50% compared to 30–35% for the wild-type protein (Fig. 5, lanes 8–10). Immunoreactive proteins of lower molecular weight were also observed in the nuclear fraction and likely correspond to cleavage products of the σ3 protein. When wild-type σ3 was coexpressed with μ1, σ3–μ1 association resulted in μ1 to μ1C cleavage (Fig. 5, lane 5). In contrast, coexpression of N16K with μ1 did not produce μ1C and, as previously reported (Mabrouk *et al.,* 1994b), there was a reduction in total accumulation of μ1–μ1C in the absence of interaction with the σ3 N16K mutant (Fig. 5, lane 11). The results also indicated that σ3–μ1 interaction occurs exclusively in the cytoplasm and that σ3 alone is transported to the nucleus; μ1 and μ1C are restricted to the cytoplasmic fraction and absent from both the cytoskeletal and nuclear fractions. The intranuclear presence of σ3 was further verified by immunoelectron microscopy using a monospecific anti-σ3 antibody. The presence of σ3 in both cytoplasmic and nuclear compartments was easily detected (data not shown). Although not strictly quantitative, these latter observations also support the idea of a selective nuclear enrichment of σ3 N16K protein.

Effect of interferon on synthesis of viral proteins in cells infected with wild-type or ts453 virus

By means of its dsRNA-binding activity, σ3 is apparently involved in translational regulation by inhibition of the cellular interferon-inducible double-stranded RNA-dependent, protein kinase (PKR) (Imani and Jacobs, 1988; Lloyd and Shatkin, 1992; Beattie *et al.,* 1995). In order to determine if the ts453 virus could further modulate PKR, L929 cells were infected at permissive and nonpermissive temperatures following interferon treatment. The multiplicity of infection (3 PFU/cell) was chosen so as to ensure infection of most cells in the culture at either temperature and the dose of interferon corresponds to nearly saturating amount on L cells (Jacobs and Ferguson, 1991; and our own unpublished data). Synthesis of viral proteins was then detected by metabolic radiolabeling followed by autoradiography. Synthesis of viral proteins was chosen as a criterion to determine the effect of interferon. This synthesis of viral protein is mostly unaffected in the thermosensitive mutant; in contrast, comparisons of viral yields between wild-type and ts viruses is rendered difficult by the decreased viral titer of this latter virus and this approach was not pursued. At nonpermissive temperature (37°C), cells infected with ts453 virus subsequent to interferon treatment exhibited no apparent decrease in synthesis of viral proteins compared to untreated infected cells; in contrast, synthesis of

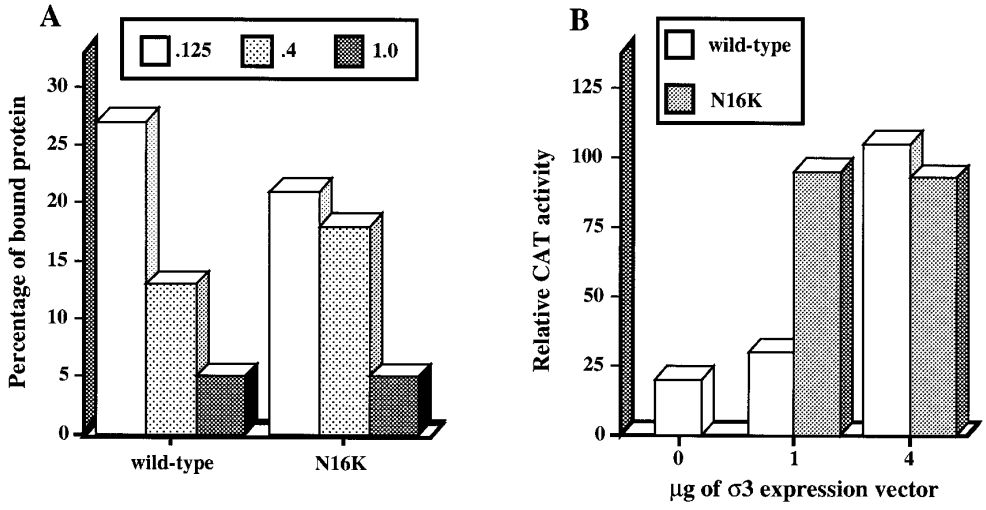

FIG. 3. Increased dsRNA binding by the σ3 N16K mutant. (A) COS cells transfected with either the wild-type or N16K expression construct were radiolabeled and proteins subjected to dsRNA affinity chromatography as described under Materials and Methods. Protein eluted at different ionic strengths were analyzed by gel electrophoresis and bound proteins quantitated relative to the original input. (B) Translational stimulation was compared by cotransfecting CAT expression vector (0.2 μg) with 1 or 4 μg of expression vector for either the wild-type or the N16K σ3 expression vector. CAT activity was quantitated by scanning of the autoradiogram following separation of acetylated products from nonacetylated chloramphenicol substrate. Results are presented as relative CAT activity compared to a control of cells transfected with 10 μg of CAT expression vector alone.

viral proteins was reduced by 25% consecutive to interferon treatment at 32°C (Figs. 6B and 6C). As usually observed, cells previously treated with interferon and infected with wild-type reovirus (T3D) exhibited an important decrease in synthesis of viral proteins at both temperatures, 50% at 37°C and 25% at 32°C (Figs. 6B and 6C). The presence of the N16K amino acid substitution thus confers a resistance to interferon specifically at the nonpermissive temperature at which expression of the corresponding phenotype is expected to occur.

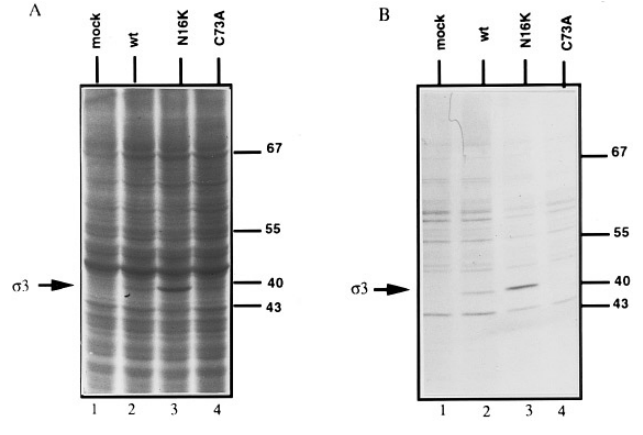

FIG. 4. Zinc-binding ability of the σ3 protein harboring the thermosensitive mutation (N16K). COS-7 cells were transfected with 10 μg of plasmid expression vector without insert (mock) or expression vectors encoding wild-type σ3 (wt), mutant N16K protein, or zinc finger mutant (C73A), as negative control. Labeled COS-7 cells were lysed and protein extracts submitted to zinc chelate affinity chromatography as described under Materials and Methods. Proteins were analyzed by SDS–PAGE followed by autoradiography. (A) Aliquots of the starting material loaded onto the zinc column; (B) proteins retained onto the column after washing at decreasing pH were eluted with acidic 0.2 M EDTA solution. The position of the σ3 proteins is indicated.

In the context of natural gastrointestinal reovirus infection, partially uncoated intermediate subviral particles (ISVPs), lacking the σ3 outer capsid protein, appear to be responsible for infection rather than complete double-shelled virions (Borsa *et al.,* 1979; Bodkin *et al.,* 1989; Bass *et al.,* 1990; Nibert *et al.,* 1991; Amerongen *et al.,* 1994). In order to determine if increased resistance observed with the ts453 virus is maintained when infection is carried out with single-shelled particles, cells were infected with ISVPs, which were generated by treatment of purified virions with chymotrypsin (Joklik, 1972; Borsa *et al.,* 1973, 1979). In addition to their lack of σ3, ISVPs can be recognized by the presence of a cleaved form of μ1–μ1C, known as the δ cleavage product. In our hands, and under the conditions used, infectious titer was not affected by chymotrypsin treatment despite the fact that virions to ISVPs conversion appeared essentially complete as judged by disappearance of σ3 and complete digestion of μ1C to δ (Fig. 6A). It was thus possible to use the same multiplicity of infection with either virus or ISVPs stocks.

The results show that the effect of interferon treatment on synthesis of viral proteins consecutive to the infection with the ISVPs derived from either wild-type or ts453 virions was the same as the effect on wild-type virions (Figs. 6B and 6C). The increased interferon resistance observed during ts453 infection is clearly lost upon removal of the outer capsid. This also provides us with a control indicating that the difference in interferon resistance observed upon infection with virions could not be

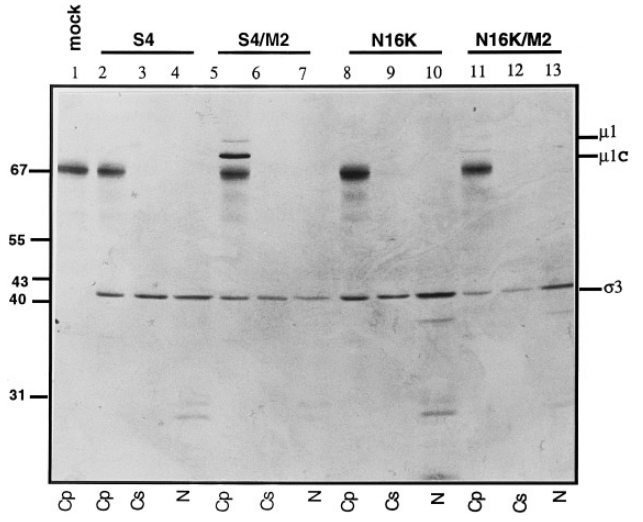

FIG. 5. Subcellular localization of the wild-type and N16K σ3 proteins. COS-7 cells were transfected with 10 μg of expression vector without insert (mock) or expression vectors encoding either wild-type σ3 (S4) or mutant N16K protein. In σ3–μ1 coexpression experiments, COS-7 cells were transfected using 5 μg of those same plasmids together with 5 μg of μ1 expression vector (M2). Cell fractionation was done according to the procedure described under Materials and Methods. Abbreviations: Cp, Cs, and N refer to the cytoplasmic, cytoskeletal, and nuclear fractions, respectively. Proteins were separated by SDS–PAGE and analyzed by immunoblotting using antireovirus antiserum. The positions of μ1, μ1C, and σ3 proteins are indicated.

due simply to a difference in number of viral particles in the inoculum.

Efficiency of uncoating of the ts453 virus

A possible explanation for the decreased interferon resistance of ISVPs could be a reduced *in vivo* uncoating efficiency of the ts453 virus in comparison to the wild-type virus (T3D). To determine whether this was actually the case, radiolabeled virions were prepared and adsorbed onto L929 cells. Infected cells were then incubated at permissive and nonpermissive temperatures to allow virus internalization and uncoating. Reovirus uncoating can be detected by the appearance of the δ cleavage product of μ1C (Sturzenbecker *et al.,* 1987; Nibert *et al.,* 1991). Analysis of viral proteins, 5 h postadsorption, revealed a similar amount of the δ cleavage product for both wild-type and ts453 viruses at permissive (32°C) or nonpermissive (37°C) temperatures (Fig. 7); in each case, between 60 to 70% of μ1C was converted to δ.

DISCUSSION

The use of thermosensitive mutants is a powerful approach in studying the function of viral proteins. Though most of them were isolated many years ago, reovirus thermosensitive mutants have remained largely unexploited. The work presented herein takes advantage of the availability of ts453 thermosensitive mutant to further support the hypothesis that the amino-terminal region of σ3 is not only involved in the interaction with μ1, but also modulates its affinity for dsRNA and, accordingly, its ability to counteract PKR action.

The present study revealed that the introduction of amino acid substitutions in the zinc finger, or introduction of the ts453 N16K substitution, increased σ3 binding to dsRNA. A similar conclusion was previously obtained by Shepard *et al.* using affinity chromatography of proteins altered in their zinc finger and expressed by *in vitro* translation (Shepard *et al.,* 1996). This also supports the idea that Northwestern blot, although using partially denatured proteins, reliably reflects the relative affinity of the proteins for dsRNA. Our Northwestern assay indicated a recovery of RNA-binding capacity for σ3 double mutants harboring amino acid substitutions within the

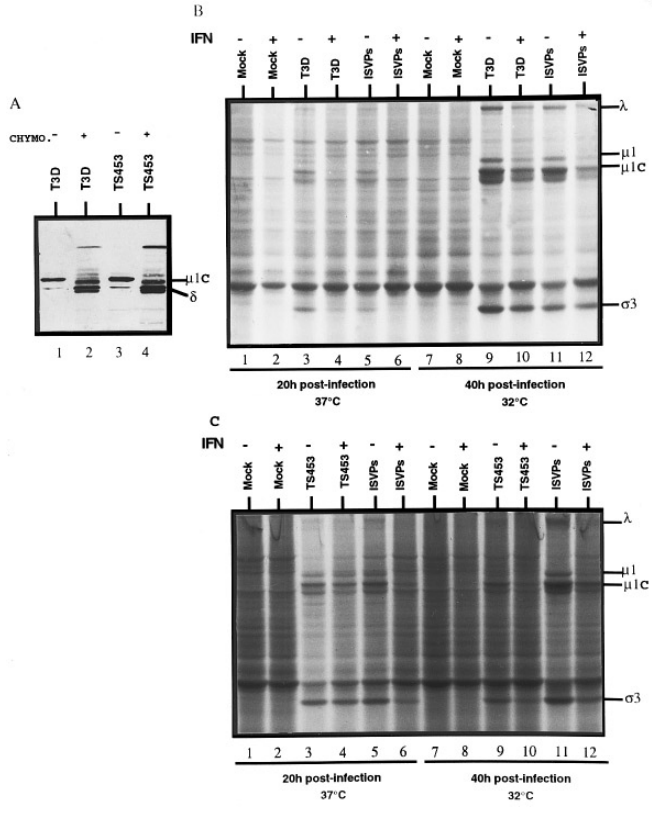

FIG. 6. Effect of interferon on synthesis of viral proteins following infection of L929 cells with virions or ISVPs. (A) Western immunoblotting using antireovirus antiserum. Samples of virions and ISVPs originating from wild-type (T3D) and ts453 viruses were generated by *in vitro* treatment of T3D and ts453 virions with chymotrypsin. The positions of σ3 and the μ1C cleavage product, δ, are indicated. (B and C) L929 cells were infected with virions or ISVPs at an m.o.i. of 3 PFU/cell in the presence (+) or the absence (−) of interferon treatment (250 IU/ml). Infections were carried out at permissive (32°C) and nonpermissive (37°C) temperatures. Viral protein synthesis was evaluated by metabolic radiolabeling followed by SDS–PAGE and autoradiography. (B) Protein synthesis following infection with wild-type (T3D) virions and ISVPs. (C) Protein synthesis following infection with ts453 virions and ISVPs. The positions of λ, μ1, μ1C, and σ3 proteins are indicated.

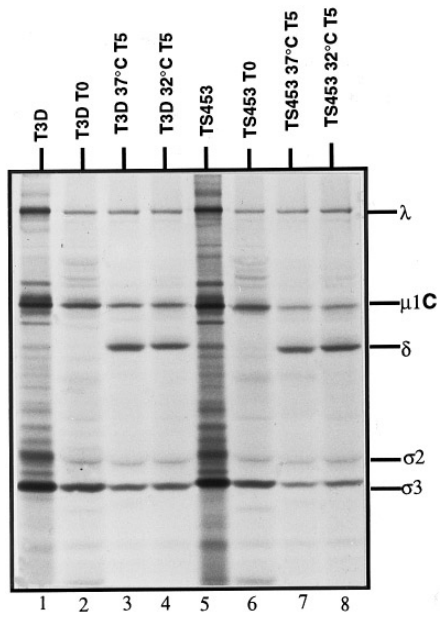

FIG. 7. Uncoating efficiency of the ts453 virus. Radiolabeled wild-type (T3D) or ts453 virus was adsorbed onto L929 cells. Radiolabeled viral proteins were then recovered immediately after the 1-h adsorption period at 4°C (T0) or after 5 h incubation (T5) at either permissive (32°C) or nonpermissive (37°C) temperature. The positions of viral proteins λ, μ1C, δ, and σ3 are indicated.

zinc finger or the N16K substitution, together with mutations affecting the first C-terminal basic motif. In addition to Northwestern analysis, binding of the N16K protein to poly(rI):poly(rC) agarose columns at higher salt concentration is another evidence for an increased ability to bind dsRNA. Finally, the better affinity of N16K for dsRNA is also supported by its increased ability to stimulate expression of the CAT gene in COS cell cotransfection experiments. Various studies have demonstrated the correlation between dsRNA binding and the ability of σ3 to stimulate translation via PKR inhibition in this assay.

The two C-terminal basic motifs are predicted to adopt an α-helical configuration using standard secondary structure prediction methods (Chou and Fasman, 1974; Garnier *et al.,* 1978). Yet, prediction algorithms indicate that the first C-terminal motif is less prone to form an α-helical structure than is the second basic motif (unpublished data). Although both basic motifs exhibit amino acid sequence similarity with other dsRNA-binding proteins (see among others: Green and Mathews, 1992; McCormack *et al.,* 1992; Chang and Jacobs, 1992), it was observed that the first motif also shares less sequence homology and displays a basic stretch which is less aligned with its analogous counterparts (Yue and Shatkin, 1996). Altogether, these findings support the idea that the first C-terminal motif can become dispensable, either upon removal of the N-terminal domain or subsequent to mutations introduced near or within the zinc finger; the first basic motif may be acting as a "buffer" to prevent the inhibitory effect of the wild-type N-terminal region. Although it is unlikely that truncated forms of σ3 could be found in replication-competent virus, it is possible that amino acid changes in the amino-terminal portion of the protein and affecting dsRNA binding and susceptibility to interferon could eventually be identified in viral isolates.

Affinity chromatography clearly showed that zinc binding is not directly affected by the N16K substitution (Fig. 3). In addition, contrary to zinc finger mutants, which are unstable when transiently expressed in COS cells (Mabrouk and Lemay, 1994a) or significantly protease-sensitive when expressed by *in vitro* translation (Shepard *et al.,* 1996), the N16K mutant was apparently stable in COS cells; its total accumulation being similar to that of wild-type. Nevertheless, it cannot be completely excluded that the σ3 produced during multiplication of ts453 virus is more unstable than the wild-type protein, since viral assembly could likely play a role in protein stability; recently published data did suggest a reduced stability of the σ3 protein during viral infection with the ts453 virus (Shing and Coombs, 1996). It thus appears that an alteration of the zinc-binding ability of the protein is not responsible for the stimulatory effect on dsRNA-binding activity, but that it is rather a conformational change exerted by amino acid substitutions within the zinc finger or surrounding this motif of the protein. In fact, it is well known that zinc fingers can be important determinants of protein conformation (see for example: McIntyre *et al.,* 1993).

The presence of σ3 in the nucleus has been recently reported and the dsRNA-binding activity of the protein was held responsible for this subcellular distribution (Yue and Shatkin, 1996). Nuclear presence of σ3 appears rather surprising considering that the nucleus does not seem to intervene during reovirus multiplication (Follett *et al.,* 1975; Zarbl and Millward, 1983), although some studies have indicated morphological alterations of the nucleus in infected cells (Chaly *et al.,* 1980). It should be mentioned that other dsRNA-binding proteins, such as the cellular PKR (RNA-dependent protein kinase) or the E3L protein of vaccinia virus (a DNA virus replicating in the cytoplasm of infected cells), were also shown to be partly localized to the nucleus (Yuwen *et al.,* 1993; Jeffrey *et al.,* 1995). In the present study, cell fractionation was used instead of immunofluorescence analysis to allow more precise determination of the relative amount of σ3 found within three distinct subcellular compartments (cytoplasm, cytoskeleton, and nucleus). The fact that σ3 N16K mutant was preferentially found in the nucleus compared to the wild-type protein further supports the importance of dsRNA-binding activity in nuclear transport. Although mechanistic details are lacking, our results with the N16K mutant are in good agreement with a model in which the affinity of σ3 is of crucial importance for nuclear presence, while blockage of this affinity, consecutive to μ1 binding, prevents this subcellular local-

ization (Yue and Shatkin, 1996). Immunoelectron microscopy of L929 cells infected with reovirus serotype 3 also revealed the nuclear presence of σ3 (unpublished data), the labeling being most intense near the nucleolus, the site of rRNA synthesis and ribosome assembly. Considering that cytoplasmic σ3 was previously found enriched in fractions prepared from infected L929 cells by high-salt wash of ribosomes (RSW) (Lemay and Millward, 1986; Lemieux *et al.,* 1987), it is tempting to speculate that the presence of σ3 in the nucleus could be somehow related to PKR inhibition.

Previous studies have indicated that the formation of σ3–μ1 protein complexes interferes with the ability of σ3 to bind dsRNA and to inhibit PKR autophosphorylation pathway. These studies include coexpression experiments in which μ1 interfered with the ability of σ3 to stimulate reporter gene expression (Tillotson and Shatkin, 1992) and the observation that σ3–μ1 complexes present in infected cells were never isolated by affinity on dsRNA columns (Huismans and Joklik, 1976; Lemieux *et al.,* 1987). The binding of σ3 with μ1 results in a conformational change rendering σ3 more sensitive to proteolytic degradation; this conformational change may be an important event conducing to the shift of σ3 from its translational control role to its structural role (Shepard *et al.,* 1995; Yue and Shatkin, 1997; Schmechel *et al.,* 1997). PKR is considered as one of the main antiviral mechanism induced by interferon and is believed to be the main intracellular factor interfering with reovirus multiplication (Wiebe and Joklik, 1975; Miyamoto and Samuel, 1980; Gupta *et al.,* 1982; Nilsen *et al.,* 1982; De Benedetti *et al.,* 1985). Our finding that the ts453 virus was resistant to the effect of interferon at nonpermissive temperature is a further evidence for the importance of σ3 in PKR regulation. It is not clear whether the increased dsRNA binding or lack of interaction with μ1 is responsible for this increased interferon resistance of ts453 virus. However, an increased dsRNA binding could be sufficient for effective PKR inhibition, since the σ3 N16K mutant was found to be more active than wild-type for the stimulation of a reporter gene in a cotransfection assay. A better affinity of σ3 for dsRNA could certainly increase its ability to inhibit PKR, especially considering that a recent report has established that this affinity is at least 50-fold lower than that of PKR (Yue and Shatkin, 1997).

Finally, the fact that ISVPs have a reduced interferon resistance suggests that viral proteins from infecting virions can play a key role in translational regulation at early stages of viral infection. A similar mechanism has been proposed for other viruses, such as mengoviruses (King and Simon, 1993), and this point certainly deserves to be further studied. Since infection by ISVPs, lacking σ3, is believed to be the main route of natural reovirus infection at mucosal surfaces (Bodkin *et al.,* 1989; Bass *et al.,* 1990; Amerongen *et al.,* 1994), our observations raise the possibility that interferon can play a greater role in the control of reovirus infection in this context.

Altogether our results demonstrate that the amino-terminal region located near the zinc-binding motif of σ3 is an essential determinant in viral protein interaction and PKR regulation through modulation of dsRNA binding. This work further stresses the importance of ts mutants for the study of viral proteins in Reoviridae. In the absence of an adequate reverse-genetics system, the ts mutants represent the best tool for correlating *in vitro* biochemical data with effects observed during the viral multiplication cycle.

MATERIALS AND METHODS

Construction of bacterial expression vectors

The cloned S4 gene encoding σ3 was subjected to site-directed mutagenesis on double-stranded DNA using the unique site elimination procedure (Deng and Nickoloff, 1992). Mutants of the amino-terminal domain were previously cloned in plasmid vectors suitable for transient expression in COS cells (Mabrouk and Lemay, 1994a), whereas mutants of the carboxy-terminal region were already available in bacterial expression vectors (Wang *et al.,* 1996). To obtain mutants harboring point mutations affecting both the amino-terminal and carboxy-terminal domains, the mutated S4 genes of the eukaryotic expression vectors were amplified by polymerase chain reaction (PCR) performed with a Gene Amp TM DNA Amplification reagent kit (Perkin–Elmer) using Ampli Taq DNA polymerase. Oligonucleotides used were 5' CCGATTGTCCATATGGAGGTGTGC and 5′ GGAACGGCTGCGCTGCGACAGTG 3'. This procedure was used to introduce a *Nde*I site at the 5' end of the S4 coding sequence, as underlined. The N-terminal fragments were then obtained following digestion with *Nde*I and *Spe*I and subcloned to replace the homologous fragments in bacterial vectors, which provided the C-terminal mutations. The final constructs were verified by direct sequencing of denatured plasmid DNA.

Protein expression and Northwestern blot assay

Bacterial expression vector pET21a and bacterial *Escherichia coli* strain BL21(DE3) were originally obtained from Novagen Inc. Plasmids harboring mutated S4 genes under the control of the T7 promoter were transformed in the BL21(DE3) strain expressing the T7 polymerase under the control of an IPTG-inducible promoter. Induction of bacterial cultures was performed as previously described (Wang *et al.,* 1996). For Northwestern blot analysis, proteins were resolved by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto nitrocellulose filters (Laemmli, 1970; Towbin *et al.,* 1979). The σ3 dsRNA-binding assay was performed using radioactive reovirus RNA (Wang *et al.,*

1996). Quantification of the autoradiograms was done with a laser personal densitometer (Molecular Dynamics). The filter was then stained with 0.1% fast green in fixing solution (40% methanol–10% acetic acid) to reveal filter-bound proteins.

Cells and viruses

Mouse L929 fibroblasts and initial inoculum of wild-type reovirus serotype 3 Dearing (T3D) were originally obtained from the American Type Culture Collection, whereas the initial inoculum of the ts453 virus was a generous gift from Dr. W. K. Joklik (Department of Microbiology and Immunology, Duke University Medical Center, Durham, NC). COS-7 cells were provided by Dr. É. A. Cohen (Département de Microbiologie et Immunologie, Université de Montréal). Viral stocks of T3D and ts453 were propagated at permissive temperature (32°C) on L929 cell monolayers, whereas L929 and COS-7 cells were maintained as previously described (Danis and Lemay, 1993; Mabrouk and Lemay, 1994b).

Transfection and metabolic radiolabeling

COS-7 cells were seeded the day before transfection at a concentration of 5×10^5 cells per 100-mm-diameter tissue culture dishes. Cells were then transfected with 10 μg of DNA using the standard calcium phosphate coprecipitation procedure (Graham and van der Eb, 1973). Expression vectors harboring an SV40 replication origin and reovirus genes under the control of SV40 promoter have been previously described (Mabrouk and Lemay, 1994b). After 16 h of contact with the precipitate, cells were subjected to three washes in phosphate-buffered saline (PBS) and fresh medium was added. For metabolic radiolabeling of proteins, cell monolayers were used 48 h posttransfection; they were washed three times in PBS and starved for 30 min in a methionine-free medium (MEM without methionine, Gibco/BRL) supplemented with 1% glutamine. Labeling was performed for 1 h with Tran^{35}S-label (ICN; 1000 Ci/mmol) at a concentration of 50 μCi/ml.

dsRNA affinity chromatography

Following metabolic radiolabeling, COS-7 cells were washed in PBS and harvested in HKM buffer [10 mM HEPES–KOH (pH 8.0), 10 mM potassium acetate, 1.5 mM magnesium acetate]. Samples were then brought to varying concentrations of NaCl before being applied onto poly(rI):poly(rC)–agarose (Pharmacia) as previously described (Mabrouk *et al.,* 1995). Proteins retained on the agarose beads following extensive washing in the same buffer were then analyzed by SDS–PAGE, autoradiography, and densitometric analysis.

Chloramphenicol acetyltransferase (CAT) assay

COS cells were transfected with either CAT expression vector alone or in combination with varying amounts of expression vector for the wild-type or the N16K mutant of σ3. In each case, the total amount of plasmid was maintained constant (10 μg per 100-mm-diameter petri dish) by adding appropriate amounts of homologous vector with no insert. Cells were lysed by repeated cycles of freezing and thawing and CAT assay was performed on cell lysates using standard procedures (Gorman *et al.,* 1982; Leahy *et al.,* 1995).

Zinc affinity chromatography

Following metabolic radiolabeling, COS-7 cells were washed in PBS and harvested in 300 μl of column buffer [50 mM Tris–HCl (pH 8.0), 150 mM NaCl] containing 0.1% NP-40. Zinc chelate affinity adsorbent (Boehringer-Mannheim) was used as described (Porath *et al.,* 1975). Briefly, samples were loaded onto a zinc affinity column and left for 2 h at 4°C. Thereafter, the column was extensively washed with buffer containing 150 and 800 mM NaCl to reduce nonspecific binding. Selective elution was performed using 0.1 M $NaPO_4$, 0.8 M NaCl with decreasing pH (7.5, 7.0, 6.5, 6.0 and 5.5), and final elution was performed with 0.2 M EDTA (pH 6.0). Protein samples were analyzed by SDS–PAGE and autoradiography. Quantification of the autoradiograms was done with a laser personal densitometer (Molecular Dynamics).

Cell fractionation and immunoblotting

Transfected COS-7 cells were recovered in PBS by centrifugation at 5000 rpm for 15 min. Cell pellets were resuspended in hypotonic buffer A [10 mM HEPES-KOH (pH 8.0), 10 mM potassium acetate, 1.5 mM magnesium acetate, and 1 mM DTT], incubated 10 min at 4°C, and centrifuged at 10,000 rpm for 10 min. The supernatants were saved as the cytoplasmic fractions. Following the same procedure, pellets were washed in buffer B [10 mM PIPES–HCl (pH 6.8), 100 mM KCl, 2.5 mM $MgCl_2$, 0.3 M sucrose, 1% Triton X-100] and resuspended in buffer C [10 mM Tris–HCl (pH7.5), 10 mM NaCl, 1.5 mM $MgCl_2$, 1% Tween 20, 0.5% sodium deoxycholate]. The supernatant obtained subsequently to the treatment with buffer C was considered the cytoskeletal fraction. Finally, the remaining pellets, composed of nuclei, were resuspended in Laemmli's sample buffer (Laemmli, 1970). The different protein fractions were analyzed by SDS–PAGE and immunoblotting using rabbit antireovirus antiserum (Lee Biomolecular) and goat anti-rabbit IgG–alkaline phosphatase conjugate (GIBCO/BRL) as previously described (Mabrouk and Lemay, 1994b).

In vitro generation of infectious subviral particles (ISVPs)

Viral stocks were prepared following infection of L929 cells in the absence of serum and were treated with 20 μg/ml of chymotrypsin (Sigma, bovine pancreas type I-S) for 30 min at 37°C. As a control, half of each viral stock was submitted to the same procedure in the absence of chymotrypsin. Viral titers of treated and untreated viral stocks were determined by using the "Tissue Culture Infection Dose 50%" method (Reed and Muench, 1938). For protein analysis, L929 cells were seeded at a concentration of 5×10^5 cells per 60-mm-diameter tissue culture dish the day before infection.

Viral infection and interferon treatment

When necessary, cells were pretreated with mouse β-interferon (Lee Biomolecular) at a saturating concentration of 250 international units (IU)/ml for 16 h. Subsequently, cell monolayers were infected at an m.o.i. of 3 PFU/cell with either ISVPs or complete virions; metabolic radiolabeling was performed 20 h postinfection at 37°C. Cells were then recovered by centrifugation and lysed in Tris-buffered saline [10 mM Tris–HCl (pH7.5), 150 mM NaCl] containing 0.1% NP-40. Labeled proteins were analyzed by SDS–PAGE followed by autoradiography. For the infections carried out at 32°C, incubation times for infection and radiolabeling were doubled.

In vivo uncoating of viral particles

Radiolabeled viral particles were obtained by infecting L929 cells with either wild-type (T3D) or ts453 viruses at an m.o.i. of 25 PFU/cell. Cells were then incubated for 12 h at 32°C in minimal Eagle's medium without methionine (MEM, Gibco/BRL). Infected cells were subsequently incubated 16 h in MEM reduced to 1/10 of normal methionine concentration and supplemented with 100 μCi/ml of Tran^{35}S-label (ICN; 1000 Ci/mmol). Viral particles were recovered by freon extraction of infected cells (Smith *et al.,* 1969) and dialyzed overnight against 100 vol of MEM. The radiolabeled viral particles were adsorbed 1 h at 4°C onto cell monolayers and removed. Cells were then washed three times in nonradioactive medium and incubated at the corresponding temperature (either 32° or 37°C) for 5 h and recovered. Viral proteins were detected by SDS–PAGE followed by autoradiography.

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