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Stoichiometry of Reovirus Structural Proteins in Virus, ISVP, and Core Particles

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All eight reovirus structural proteins were resolved in a new tris, glycine, and urea (TGU) electrophoretic gel system. The specific identities of proteins were determined immunologically, biochemically, and genetically. Structural proteins of reovirus type 1 Lang had different mobilities in the TGU gel than did type 3 Dearing proteins. Intertypic reassortant viruses that contained various combinations of parental genes were used to identify each of the viral protein bands. Type 1 Lang virions were metabolically-labelled with either ^{3}H-amino acids or ^{35}S-methionine/cysteine and gradient purified. Aliquots of purified virions were treated to generate infectious subviral particles (ISVPs) and core particles. Radiolabelled virus, ISVP, and core proteins were resolved in the TGU gel and protein band intensities were used to determine copy numbers of each structural protein. These studies confirmed the copy numbers and locations of most reovirus proteins. However, important new findings include the discovery that virions contain approximately 120 copies of major core protein σ2 and 20 copies of the polymerase cofactor protein μ2, and ISVP particles contain about 24 copies of μ1C that has not been processed to the δ peptide. These data are used to generate a new model of the arrangement of structural proteins within the reovirus particle.

INTRODUCTION

The mammalian reoviruses are the prototypic members of the orthoreovirus genus of the family *Reoviridae.* This family includes a number of important human (rotavirus) and animal (orbivirus) pathogens. Reovirus has a genome of 10 double-stranded (ds) RNA segments which is enclosed in a double shell of protein (for reviews see: (Nibert *et al.,* 1996b; Tyler and Fields, 1996)). The innermost capsid shell (called the core) is a multienzyme complex that contains all necessary components for transcription, methylation, and capping of progeny messenger RNA from the enclosed genome (Chang and Zweerink, 1971; Shatkin, 1974; Furuichi *et al.,* 1975). Cores are composed of multiple, but non-equivalent, copies of five proteins: λ1, λ2, λ3, μ2, and σ2. The outermost capsid is composed of multiple copies of three additional proteins: μ1 (and/or its cleavage product μ1C), σ1, and σ3. Recent electron cryomicroscopy image reconstructions clearly reveal that the outer capsid is made up of 600 copies each of proteins μ1/μ1C and σ3 (Dryden *et al.,* 1993). Biochemical studies have indicated that there are 60 copies of core spike protein λ2 (White and Zweerink, 1976) and that the cell attachment protein σ1 is a trimer (Strong *et al.,* 1991). Protein σ1 trimers are located at the vertices of the viral particle in association with the λ2 spikes (Furlong *et al.,* 1988), implying the presence of 36 molecules of σ1 in each viral particle. However, all 12 icosahedral vertex positions may not be occupied by the cell attachment protein (Larson *et al.,* 1994). Estimates of the copy numbers of other major structural proteins also have been reported (Smith *et al.,* 1969).

Despite the significant amount of detail known about the structure, pathogenesis, and some aspects of replication of reovirus, little is known about how mRNA is transcribed from the genomic RNA, and about how the mRNA is subsequently used to make progeny double-stranded RNA. Recent evidence indicates that these enzymatic functions are mediated by two core proteins that are present in small copy number. Protein λ3 is associated with the pH optimum of transcription (Drayna and Fields, 1982), contains sequence motifs present in other RNA polymerases, including the GDD motif (Wiener and Joklik, 1989; Morozov, 1989; Bruenn, 1991), and recombinant λ3 has poly(C) dependent poly(G) polymerase activity (Starnes and Joklik, 1993). Protein μ2 has been associated with the temperature optima of both *in vitro* transcription (Yin *et al.,* 1996) and *in vitro* nucleoside triphosphatase acitivity (Noble and Nibert, 1997), and studies of a temperature-sensitive mutant that maps to this protein also suggest μ2 is involved in RNA synthesis (Coombs, 1996). However, the precise locations and numbers of copies of these important proteins remain unknown. Recent electron cryomicroscopy reconstructions of genome-deficient "top component" particles suggest λ3 may be located near the vertices of the particle (Nibert and Dryden, personal communication, 1998). In light of increased structural information that is being provided both by electron cryomicroscopy (Dryden *et al.,* 1993; Nibert and Dryden, personal communication, 1998;

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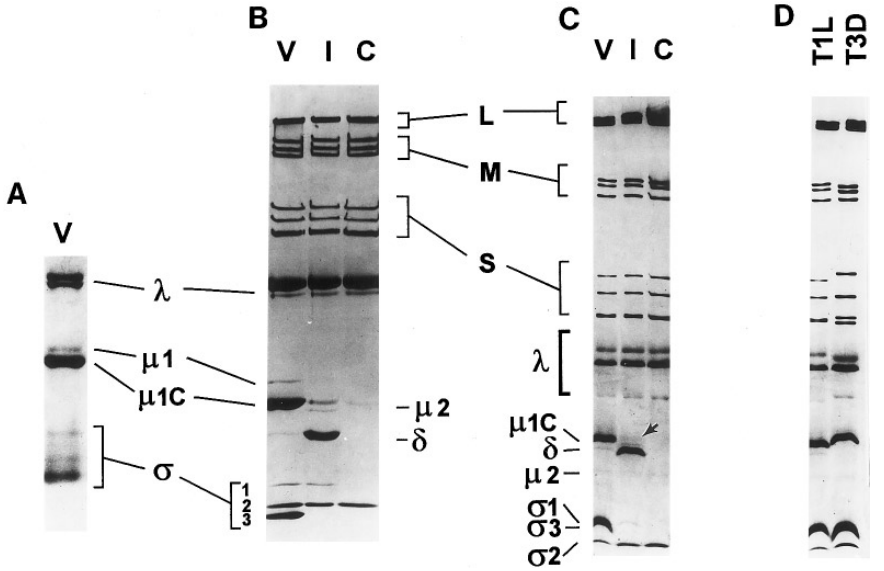

FIG. 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of reovirus proteins. About 3×10^{11} of indicated types of particles [Type 1 Lang (T1L) virions (V), ISVPs (I), and cores (C); and type 3 Dearing (T3D) virions] were resolved in either (A) a 5-10% linear gradient phosphate-urea gel as described (Summers *et al.,* 1965); (B) a 4-16% exponential gradient discontinuous tris-glycine gel (Laemmli, 1970); or (C) and (D) 4-16% exponential gradient tris-glycine-urea (TGU) gels (as described in Methods). After electrophoresis, gels were fixed in 30% isopropanol/10% acetic acid, silver stained (Merril *et al.,* 1981) to visualize RNA (B-D; indicated by "L", "M", and "S" and brackets between panels B and C), and then stained with Coomassie Brilliant Blue (A–D). The identities of viral proteins are indicated between panels A and B, and the identities of those proteins and peptides seen in ISVPs and cores are indicated to the right of panel B. Assignment of identities of some proteins in the TGU gel (indicated to left of panel C) is based on the appearance, or loss, of these proteins in the various intact and subviral forms of the virus. Note the presence of a small amount of material that migrates as intact μ1C in the ISVP lane (panel C, I lane, arrow; discussed in the text). Proteins σ1 and σ3 were not well resolved in this particular gel.

Yeager, Weiner, and Coombs, in preparation) and X-ray crystallography (Coombs *et al.,* 1990), and increased interest in the functions of these proteins (for examples, see: (Matoba *et al.,* 1993; Starnes and Joklik, 1993; Cashdollar, 1994; Sherry and Blum, 1994; Coombs, 1996), the present study was undertaken to determine the copy numbers of each of these enzymatic proteins.

RESULTS AND DISCUSSION

Resolution of all reovirus structural proteins in hybrid tris-glycine-urea (TGU) sodium dodecyl sulfate-polyacrylamide gel electrophoresis SDS-PAGE

Reovirus proteins have been, and are still, usually resolved in either "phosphate-urea" gels (Summers *et al.,* 1965) or in "Laemmli" discontinuous tris-glycine gels (Laemmli, 1970). Neither is capable of resolving all virion structural proteins. The phosphate-urea gel system, which was the first employed (for example, see: (Smith *et al.,* 1969)), was capable of resolving most of the virion proteins (Fig. 1A). However, the minor core proteins λ3 and μ2 could not be resolved from λ1 and μ1C, respectively. In addition, this continuous-buffer type of gel system provided serious limitations, both in the amount of material that could be loaded, and in the speed at which the gels could be electrophoresed. Both limitations were removed by the subsequent development of the "Laemmli" type of gel (Laemmli, 1970). While the Laemmli type of gel has proven invaluable to most investigators, a serious problem remained for reovirologists interested in separating every protein. Although the λ3 protein could now be resolved, the largest major core proteins, λ1 and λ2, usually could not be resolved from each other (Fig. 1B). This problem has been solved by altering the concentration of bisacrylamide cross-linker (Cleveland *et al.,* 1986; Nibert *et al.,* 1996a). However, the μ2 core protein still could not be resolved from major outer capsid protein μ1C in these tris-glycine gel systems. Because the present study was aimed at better characterizing the structures of the various forms (intact and subviral) that the virus manifests during the replicative cycle (assembly and disassembly), it was necessary to accurately determine the number of copies of *all* structural proteins.

In an attempt to accomplish resolution of more structural proteins, components of the phosphate-urea and Laemmli-type gels were combined to generate a hybrid resolving gel system. A number of different hybrid systems were tested. The one that gave optimum separation of reovirus proteins consisted of a 4cm step of 4% SDS-PAGE (using the tris-glycine (Laemmli) system which contains no urea) placed on top of a 14cm tall 4-16% exponential gradient SDS-tris-glycine-PAGE that contained 7M urea. A total of eight distinct protein bands were observed when virus proteins were resolved in this hybrid tris-glycine-urea (TGU) gel (Fig. 1C, lower half).

This is a greater number of protein bands than that obtained in either of the two systems from which the hybrid was derived. The presence of eight distinct protein bands suggested that each of the eight virion structural proteins had been resolved in this hybrid gel. The eight protein bands resolved as a spectrum between small and large sized, rather than falling into three distinct size classes as was the case for resolution of the proteins in the standard tris-glycine system. The identities of each of the eight protein bands was determined both genetically and biochemically. The identities of some of these proteins could be deduced by comparing the pattern of selective loss, or appearance, of protein species as the virus was digested to ISVPs and cores (compare panel B to panel C). For example, the major protein band present near the bottom of the gel that was present in virions but absent in ISVPs can be identified as σ3; and the conversion of an intense medium-molecular weight band in the virus sample to an intense band of slightly smaller molecular weight in the ISVP (and its loss upon subsequent conversion to cores) identifies the two bands as μ1C and δ, respectively. Proteins identified in this manner are indicated to the left of the gel (Fig. 1C). The migration order of the two smallest virus proteins (σ2 and σ3) was altered by the presence of high concentrations of urea in the resolving gel (data not shown).

In order to complete the identification of various protein bands in the TGU gel, (particularly λ2, which would be useful as an internal standard for protein copy number determinations because its copy number is known to be 60 (White and Zweerink, 1976; Dryden *et al.,* 1993) and because it is present in virions, ISVPs, and cores), I took advantage of the observation that most of the proteins from type 1 Lang (T1L) and type 3 Dearing (T3D) showed electrophoretic mobility differences in this hybrid gel system (Fig. 1D). Our lab, and others, have used reassortant reoviruses to map strain-specific differences to specific viral gene(s) (for examples, see: (Weiner *et al.,* 1980; Drayna and Fields, 1982; Keroack and Fields, 1986; Bodkin and Fields, 1989; Matoba *et al.,* 1991; Yin *et al.,* 1996)). Each of the gene → protein coding assignments are known: L1 → λ3, L2 → λ2, L3 → λ1, M1 → μ2, M2 → μ1/C, S1 → σ1, S2 → σ2, and S4 → σ3 (M3 and S3 encode non-structural proteins) (McCrae and Joklik, 1978; Mustoe *et al.,* 1978). Therefore, reovirus reassortants have proven useful for determining which proteins are responsible for particular phenotypes. The same approach was used to determine the identities of each of the proteins that had been resolved in the TGU gel. Reassortant viruses were grown, purified, and dissolved in electrophoresis sample buffer as described above. Virion RNAs were resolved in 10% SDS-PAGE (Sharpe *et al.,* 1978; Hazelton and Coombs, 1995) and proteins were resolved in TGU gels (Fig. 2). Proteins were identified by correlating strain-dependent RNA mobility differences with strain-dependent protein mobility differences. For example, the difference seen between T1L and T3D in the mobility of the uppermost protein band in most clones (Fig. 2B) mapped to the L2 gene (compare to Fig. 2A). Therefore, this protein could be identified as the λ2 protein. Similarly, the difference in migration of the protein band identified as λ3 correlated with the difference in mobility of the L1 gene segment. Many of the remaining protein bands were identified in this manner and their identities are indicated to the left of the gel. Electrophoretic mobility differences were not seen reproducibly with either λ1 or μ2. Their identifications were based on exclusion after assignment of the other six structural proteins, by their selective presence in various particles (above), immunologically, and biochemically (see below).

One potential concern involved the identification of λ3 and μ2 (the minor core proteins this study was initially focussed upon as explained above) because their migrations in the TGU gel system were considerably faster than might have been expected, based on their migration rates in either the phosphate-urea or Laemmli gel systems. This abnormal migration behavior might have been caused by any of three circumstances. The first possible explanation is that the mobilities of these proteins were significantly affected by the presence of urea in the tris-glycine based buffers. The second possible explanation is that these bands might represent other peptides, the mobilities of which were affected by the L1 and M1 gene products, thereby explaining the mapping of mobility differences to these gene segments. To test the second possibility, virion proteins were resolved in a TGU gel, transferred to Immobilon membranes, and probed with various monospecific polyclonal antisera. The band identified as "λ3" was the only protein recognized by an anti-λ3 antiserum (generously provided by Dr. W. Cashdollar) and the band identified as "μ2" was the only protein recognized by an anti-μ2 antiserum (generously provided by Dr. E. Brown) (data not shown). The final possible explanation for the aberrant mobilities is that the "λ3" and "μ2" bands might represent cleavage products of these respective proteins. Such protein alterations would affect interpretations of protein stoichiometry that are based on band intensity and molecular weight (see below). Therefore, to determine the intactness of these proteins, as well as biochemically confirm each protein's identification, ^{35}S-Met/Cys-labelled T1L was grown and purified as described above. Radiolabelled proteins were resolved in the TGU, phosphate-urea, and Laemmli-type gels. Individual radiolabelled proteins were excised. Comparisons of *S. aureus* V8 protease and tryptic digest patterns (prepared as described (Cleveland *et al.,* 1977)) of protein bands excised from the three types of gels confirmed each protein's identification (data not shown). In addition, sets of gel slices from the different types of gels were re-electrophoresed in another Laemmli-type gel. The excised and re-electrophoresed bands migrated at the same rate as

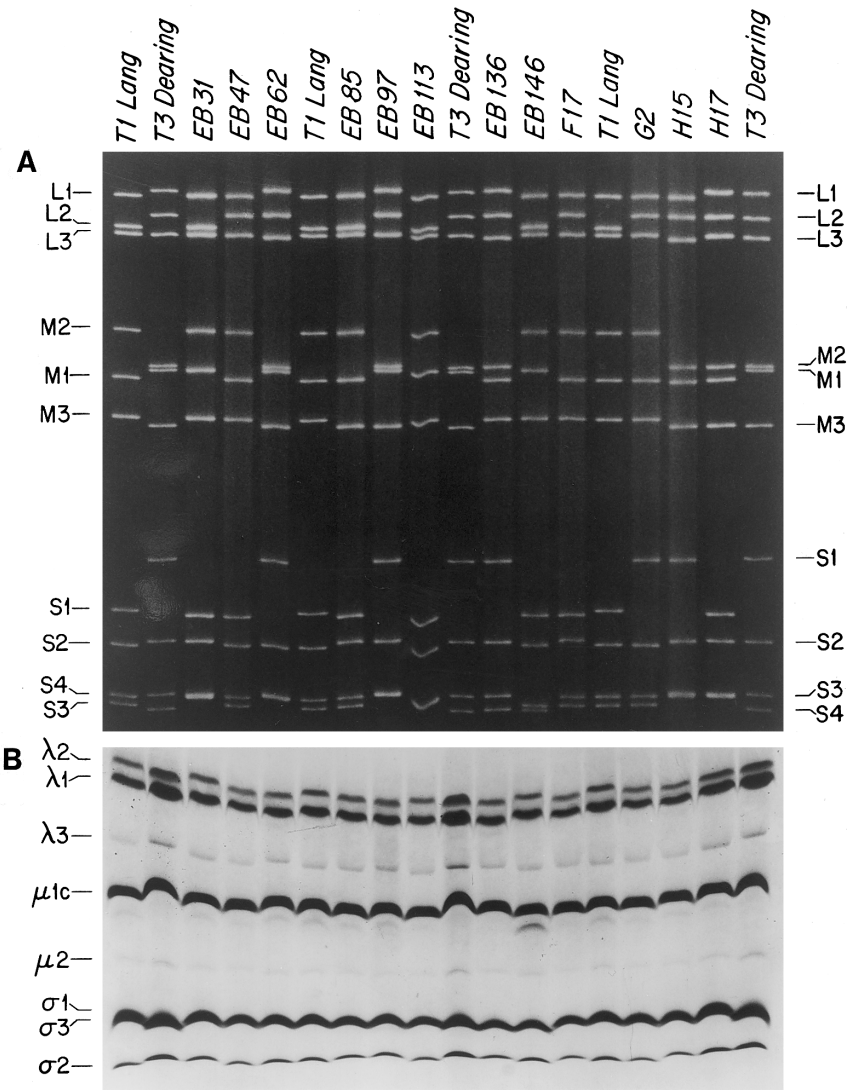

FIG. 2. SDS-PAGE of T1L, T3D, and various T1L × T3D intertypic reassortant viruses. (A) About 7×10^{10} purified particles of each virus clone (indicated above lanes) were electrophoresed in a 10% gel (16.0 × 16.0 × 0.15cm; 18mA for 45h) to resolve viral gene segments which were stained with ethidium bromide and visualized under ultraviolet light. The identities of each gene segment are indicated to the left (T1L) or to the right (T3D) of the gel. (B) About 2.5×10^{11} purified particles of each virus (as indicated above in "A") were electrophoresed in a TGU gel to resolve viral structural proteins and stained with Coomassie Brilliant Blue. The identities of most proteins were determined by comparison of the observed electrophoretic differences of each with the electrophoretic differences seen in the gene segments in (A) and are indicated to the left of the gel. Proteins σ1 and σ3 (particularly those of T3D) were not well resolved in this particular gel.

appropriate marker virus proteins, indicating no loss of molecular mass from the proteins that had been resolved in the TGU gel (Fig. 3). This analysis indicated that the proteins that had been resolved in the hybrid gel were intact and that stoichiometric analyses based on band intensity and molecular weight would be accurate.

Stoichiometric determination of proteins in virus, ISVPs, and cores

Stoichiometric determinations of viral protein copy numbers were carried out on T1L because this strain showed greater resolution of the various structural proteins than did T3D (Fig. 1D). In particular, there was better, and more reproducible, separation between the T1L λ1 and λ2 proteins than there was between the T3D λ1 and λ2 proteins. This is important because protein λ2 is the only protein common to virions, ISVPs, and cores and for which the copy number is unequivocally known (White and Zweerink, 1976; Dryden *et al.,* 1993); thus, it would be useful as an "internal reference". In addition, T1L virus, and its subviral forms, are generally more stable than are those derived from T3D (Dryden *et al.,* 1993). Uniform labelling of virus proteins was achieved by labelling T1L-infected cells with ^{3}H-amino acids mixture. In addition, virus metabolically labelled in methionine and cysteine residues was purified. Both labelling

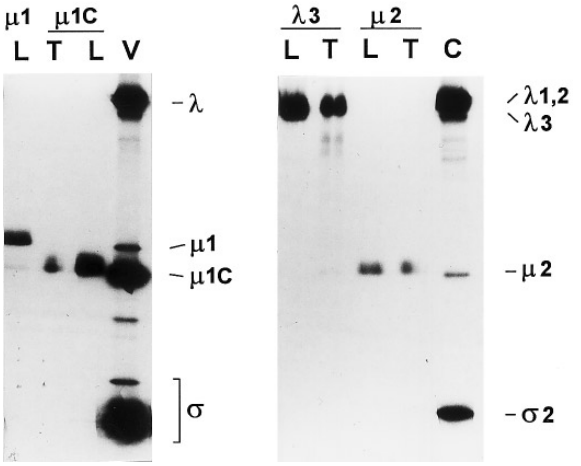

FIG. 3. Re-electrophoresis of isolated reovirus protein bands. Aliquots of 1.4×10^{10}–1.1×10^{12} [^{35}S]-Met/Cys-labelled virion and core particles were resolved in a standard "Laemmli" (L) or TGU (T) gel. Protein bands were lightly stained to locate them, bands corresponding to about 1×10^{13} molecules (based upon previous copy number estimates (Nibert *et al.,* 1996b)) excised, and then prepared for re-electrophoresis in a 4-16% exponential gradient Laemmli gel. The gel was fixed, impregnated with Enlightning, dried, and exposed to Kodak X-Omat AR X-ray film. Putatively identified viral proteins are indicated above corresponding lanes. (V) Marker virus sample; (C) marker core sample, with proteins identified.

regimens were used in order to employ both laser film densitometry and phosphorimaging techniques and because some of the T1L gene and protein sequences are currently not available. Virus and subviral particles were purified and dialyzed as detailed below in Materials and Methods.

The structural proteins of radio-labeled virus, ISVPs, and cores were resolved in hybrid TGU gels (Fig. 4), and, for comparative purposes, in "standard" 5-15% linear gradient Laemmli SDS-PAGE. Gels that contained ^{3}H-uniformly-labeled virus proteins were fixed, impregnated with Enlightning (DuPont, Mississauga, Ontario), dried, and fluorographed (Kodak X-AR X-ray film, Rochester, NY) (Hames and Rickwood, 1981). Multiple film exposures of the gels were made and scanned with an LKB Ultroscan XL laser densitometer. Band intensities were scaled to each film exposure to determine the range of linear film response for each sample and the amount of material present in each band. The amount of protein present in each band in each particle type was calculated by scaling band intensities in each lane to a value of 60 λ2 molecules (present as about 52 copies of intact λ2 and 8 copies of λ2C) in the same lane. Thus, 52 copies of the 1289-amino-acid long λ2 protein accounts for 67,028 total λ2 amino acids per particle. Linearization of multiple gel exposures of multiple sample preparations indicated that in intact virus the μ1C band was 6.34–6.66 times as intense as the λ2 band, and the σ3 band was 3.22–3.44 times as intense as the λ2 band. The intensity of the μ1C band predicts that each virion contains about 435.7 (± 10.7) $\times 10^{3}$ μ1C amino acids. The μ1C protein contains 666 amino acids. Thus, the μ1C band intensity predicts that each virion contains 621 (± 15) copies of μ1C (Table 1, leftmost 4 columns), a value that is in close agreement with electron cryomicroscopy-derived values of 600 (Dryden *et al.,* 1993). The calculated errors associated with each value represent differences obtained from multiple particle preparations and multiple gel analyses. Likewise, the intensity of the σ3 band predicts that each virion contains about 223.3 (± 7.6) $\times 10^{3}$ σ3 amino acids. The σ3 protein contains 365 amino acids. Thus, the σ3 band intensity predicts that each virion contains 617 (± 21) copies of σ3 (Table 1, leftmost 4 columns), a value that also is in agreement with electron cryomicroscopy-derived values of 600 (Dryden *et al.,* 1993). Similar calculations were performed for each of the other proteins in all three types of particles (Table 1, left half).

Protein copy numbers also were deduced from ^{35}S-labelled particles. Proteins were resolved in gels as described above and radioactivity captured and measured with a Molecular Dynamics PhosphorImager. In this case direct counts for each protein band were scaled to a λ2 value of 2236 (the predicted total number of methionine and cysteine residues present in 52 copies of λ2). Preliminary experiments suggested that the amount of acrylamide (as a function of both gel concentration and gel thickness) in the lower portions of the TGU gel (corresponding to the exponential concentration region) contributed to significant signal quenching. Therefore, it was necessary to resolve samples in thinner (0.75mm) TGU gels to determine the relative copy numbers of the larger λ and μ proteins, and in a series

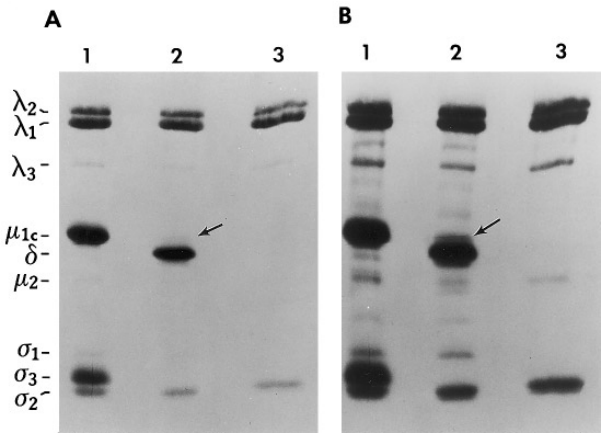

FIG. 4. SDS-PAGE analysis of ^{3}H-labeled T1L virus (1), ISVPs (2), and cores (3). About 7.5×10^{11} of each type of gradient-purified particle were resolved, in alternate lanes, in a TGU gel. The gel was fixed, impregnated with Enlightning, dried, and exposed to Kodak X-Omat AR X-ray film. Two different exposures of the same gel are shown (A and B). Material in each band was measured, scaled, and protein copy numbers calculated (shown in Table 1, columns 4-6). Note the presence of a small amount of material that migrates as intact μ1C in the ISVP lanes (arrow).

TABLE 1

Molecular Weights, Copy Numbers, and Locations of Structural Proteins in T1L Reovirus

		All amino acid residues (^{3}H)[a]				Methionine and cysteine residues (^{35}S)[b]			
		Number amino	Copy number in			Number methionine	Copy number in		
Protein	kDa[c]	acids[c]	Virus	ISVP	Core	and cysteine[c]	Virus	ISVP	Core
λ1	137.4†,[d]	1233†,[d]	124 ± 3	122 ± 3	123 ± 3	55†	121 ± 4	118 ± 5	119 ± 4
λ2	167.0†	1289†	60[e]	60[e]	60[e]	43†	60[e]	60[e]	60[e]
λ3	142.4	1267	13 ± 1	12 ± 1	11 ± 1	66	12 ± 1	11 ± 2	12 ± 2
μ1C	72.1	666	621 ± 15	23 ± 3	ND[f]	19	597 ± 9	29 ± 3	ND
δ	58.8	539	ND	575 ± 16	ND	17	ND	570 ± 5	ND
μ2	83.3	736	21 ± 3	17 ± 4	14 ± 3	37	18 ± 3	18 ± 4	19 ± 3
σ1	51.4	470	37 ± 3	23 ± 2	ND	12	39 ± 5	28 ± 5	ND
σ2	47.1	418	122 ± 12	126 ± 12	117 ± 15	18	114 ± 16	104 ± 17	117 ± 15
σ3	41.2	365	617 ± 21	ND	ND	27	618 ± 31	ND	ND

[a] Determined from densitometric scans of X-ray films of ^{3}H-uniformly-labelled virus proteins (see text for details).

[b] Determined by phosphorimaging scans of [^{35}S]methionine/cysteine-labelled virus proteins (see text for details).

[c] Predicted molecular weights (column 2); and number of total amino acids (column 3), or of methionine and cysteine residues (column 7), in each protein, as determined from sequence predictions; λ1, (Bartlett and Joklik, 1988); λ2, (Seliger *et al.,* 1987); λ3, (Wiener and Joklik, 1989); μlC, δ, (Wiener and Joklik, 1988; Nibert and Fields, 1992); μ2, (Zou and Brown, 1992); σ1,(Nibert *et al.,* 1990); σ2, (Dermody *et al.,* 1991); σ3, (Atwater *et al.,* 1986).

[d] † , sequence not currently available for T1L; value shown is deduced from known T3D sequence.

[e] Value determined in (White and Zweerink, 1976; Dryden *et al.,* 1993) and used as a standard to calculate other protein copy numbers within the same sample (see text for details).

[f] ND, none detected.

of linear concentration SDS-PAGE to determine the relative copy numbers of the σ proteins. Calculations similar to those described in the previous paragraph, but scaling to methionine and cysteine residues rather than to total amino acids, were performed for each of the proteins in each of the particle types (Table 1, right half). Values generated by phosphorimage analyses of ^{35}S-labelled particles were generally in good agreement with values generated by linearized densitometric film analyses of ^{3}H-labelled particles. The combination of these analyses indicated that each type of particle contains approximately 120 copies each of λ1 and σ2 (Table 1). Virions also contain about 12 copies of λ3 and about 36 copies of σ1. Most of these values correspond well with previously published data (Smith *et al.,* 1969; Strong *et al.,* 1991) and estimates (Nibert *et al.,* 1996b). The 37-39 copies of σ1 found in T1L virions is significantly higher than the number of copies found in T3D virions (Larson *et al.,* 1994) and probably represents strain-specific differences in structural stability (discussed above). Virions also contain approximately 18-20 copies of μ2, a value that differs from previous estimations. These differences probably reflect the inability to resolve this protein in the other gel systems. The value of almost 24 copies of μ2 might indicate that two copies of this polymerase cofactor protein (which presumably interacts with other proteins such as λ2 and λ3 which are involved in RNA transcription, capping, and extrusion) are located near, or at, each of the 12 particle vertices. Alternatively, the value of 20 copies might mean two copies of μ2 are associated with each of the 10 gene segments. However, each of these proteins are also present at about the same copy number in genome-deficient top component particles (data not shown), indicating that these proteins are primarily associated with the protein capsids.

During the course of this study, and as a result of the available deduced molecular masses of all structural proteins and the present determination of copy numbers of each protein in each type of viral and subviral particle, it was possible to re-evaluate the extinction coefficient of all particle types. The aggregate molecular mass of protein within a virion particle (number of copies of each protein times its molecular mass - from appropriate columns in Table 1) can be calculated as 106.4 MDa (Table 2), in close agreement with previous dry weight determinations of 109 MDa (Farrell *et al.,* 1974). The mass of protein within a core particle can be calculated as 35.2 MDa, also in close agreement with a dry weight determination of 37.3 MDa (Farrell *et al.,* 1974). The mass of protein within an ISVP was calculated as 80.4 MDa. When combined with the mass of RNA present in each type of particle, total particle masses of 125.7 MDa for virus, 97.3 MDa for ISVPs, and 50.2 MDa for cores were obtained. These values agree well with previous ultracentrifugation-derived (for virus and cores) values of 129.5 MDa and 52.3 MDa respectively (Farrell *et al.,* 1974), and with recent scanning transmission electron microscopic (STEM) mass measuremnents of 128.6 MDa for virus, 103.1 MDa for ISVPs, and 53.1 MDa for cores (Wall, Hainfeld, Furlong, and Coombs, unpublished).

TABLE 2

Predicted Total Particle Masses of Reovirus and Subviral Particles

	Particle		
	Virus	ISVP	Core
Total protein mass (MDa)[a]	106.4	80.4[b]	35.2
Mass of RNA[c]	19.3	16.9 (±2.4)	15.0
Calculated total particle mass (MDa)	125.7	97.3	50.2

[a] Calculated by summing the products of all protein copy numbers and molecular weights (from Table 1).

[b] Calculated by summing the products of all protein copy numbers and molecular weights (from Table 1), and including predicted mass of 575 copies of protein θ (not shown in Table 1 but present in particle).

[c] From Joklik, 1983.

When calculated aggregate protein masses are combined with the value that 1 ODU_{260nm} corresponds to 2.1×10^{12} virion particles per milliliter (Smith *et al.,* 1969), this suggests that 1 ODU_{260} corresponds to a calculated value of about 0.37mg virus protein per milliliter. Comparisons of optical density, specific radioactivity, and protein band intensity measurements of each type of viral and subviral particle were used to calculate extinction coefficients for each of the other types of particles that may be obtained from either purified virions or genome-deficient top component particles (Table 3).

Model of protein packing within reovirus

The newer protein copy numbers, determined as described above, were used to generate a new model of protein packing within the reovirus particle (Fig. 5). Within the virion particle the locations and relative orientations of outer capsid proteins μ1 and μ1C, σ1, and σ3, and core spike pentamers of λ2 have been previously determined (Luftig *et al.,* 1972; Hayes *et al.,* 1981; Furlong *et al.,* 1988; Metcalf *et al.,* 1991; Dryden *et al.,* 1993). The precise locations of the other core proteins remain to be elucidated. Proteins λ1 and σ2 are the major core structural proteins, which together, constitute the core capsid (Xu *et al.,* 1993). Minor core proteins λ3 (and presumably μ2) are internal (Cashdollar, 1994), and, considering their primarily enzymatic roles, may be located near the λ2 spikes which possess capping functions (Cleveland *et al.,* 1986; Mao and Joklik, 1991). Conversion of the virus to the ISVP results in several well-known alterations: the ISVP lacks detectable σ3, as previously reported (Smith *et al.,* 1969; Nibert *et al.,* 1996b), σ1 takes on an extended form (Furlong *et al.,* 1988), and μ1C is proteolytically converted to the δ and θ peptides, both of which remain associated with the particle (Nibert and Fields, 1992). The calculations in this report indicate that T1L ISVPs contain about 23-28 copies of intact σ1. This is fewer than the number found in the whole virion and probably reflects susceptibility of these extended fibers (Furlong *et al.,* 1988) to shear forces during purification, but significantly greater than the number of intact σ1 proteins found in T3D ISVPs (Nibert *et al.,* 1995). Surprisingly, the ISVP still contains about 23-29 copies of a protein that migrates as μ1C, although the vast majority of this outer capsid protein has been converted to the δ peptide. These non-cleaved μ1C proteins were consistently found in many different particle preparations and remained despite prolonged (up to 4h) protease treatment (data not shown), suggesting that they are not an artifact of purification. The locations of these non-cleaved μ1C proteins are currently unknown. A value close to 24 might indicate that 2 copies are present at each of the 12 icosahedral vertices. Alternatively, these non-cleaved μ1C proteins may be clustered around one, or a few, of the vertices. Several characteristics of the μ1C and σ3 proteins, and the way they interact with each other, might explain the observation that a small number of μ1C proteins are not proteolytically processed in the ISVP particle. Kinetic studies indicate that σ3 is nearly completely removed before μ1C is processed to δ (Nibert and Fields, 1992; Nibert *et al.,* 1995; Coombs, unpublished). Thus, it is likely that the trypsin and chymotrypsin cleavage sites in μ1C are masked by σ3 within the particle. The presence of λ2 within some outer capsid preparations (Matsuhisa and Joklik, 1974), as well as direct electron cryomicroscopy observations (Dryden *et al.,* 1993), indicate significant interactions between λ2, μ1C, and σ3. Finally, the intercallation of μ1C and σ3, and presence of equivalent copy numbers of each within the λ2 framework (Dryden *et al.,* 1993), imposes sidedness to the μ1C/σ3 interaction. This imposed μ1C polarity suggests that a small number of μ1C proteins that are adjacent to a λ2 spike may have their μ1C → δ/θ cleavage site (indicated by "✶" in ISVP portion of Fig. 5) masked by λ2 rather than by σ3. Thus, these few μ1C

TABLE 3

Relationship between 1 ODU_{260} and Quantity of Various Purified Reovirus Viral and Subviral Particles

Type	Number particles[a]	Protein[b]
Virion[c]		
Virus	2.1×10^{12}	0.37
ISVP	2.7×10^{12}	0.36
Core	4.4×10^{12}	0.26
Top component[d]		
Virus	8.7×10^{12}	1.54
ISVP	1.2×10^{13}	1.60
Core	2.1×10^{13}	1.23

[a] Number of indicated types of particles per optical density unit at 260 nm per milliliter.

[b] Expressed as total calculated amount of protein (in mg) per ODU_{260} per milliliter.

[c] Particles that contain genome.

[d] Particles lacking genome.

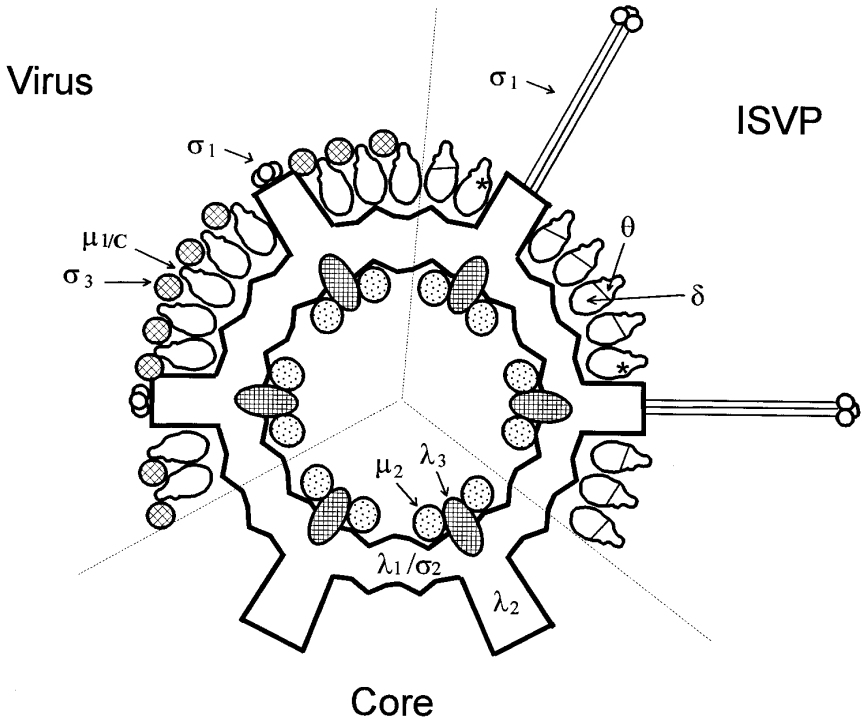

FIG. 5. Model for protein packing in reovirus virion, ISVP, and core particles. Examples of proteins associated primarily with the virion form of the particle [μ1 and μ1C (designated μ1/C), retracted σ1, and σ3] are indicated in the upper left portion of the figure. Examples of proteins found exclusively in the ISVP form of the virus (extended σ1, δ, and θ) are indicated in the upper right portion of the figure. The cleavage site in μ1C that generates δ and θ that is suggested to be blocked by proximity to λ2 is indicated by "✱". The core proteins are indicated in the bottom portion of the figure. The difference in shape of λ2 in the core particle as compared to its conformation in the other two particles represents the previously described conformational change of this protein associated with transition to the core (Dryden *et al.*, 1993).

proteins may remain intact, even after removal of σ3. It seems likely that these intact μ1C were not previously detected because of the co-migration of μ1C and μ2 in earlier gel systems. Retrospective analyses of the quantities of virion, ISVP, and core proteins resolved in the "standard" Laemmli-type of SDS-PAGE reveal that in most cases, the μ1C/μ2 band in ISVPs is more intense than is the μ2 band in cores (based upon equivalent particle loadings), suggesting the presense of extra material, and indicating that the presense of uncleaved μ1C in ISVPs is not an artifact of the newer TGU system (for example, compare Fig. 1B, 'I' and 'C' lanes). The role(s), if any, that these non-cleaved μ1C proteins play in virus entry into cells remains to be elucidated.

MATERIALS AND METHODS

Cells and viruses

Reovirus serotype 1, strain Lang (T1L), serotype 3, strain Dearing (T3D), and T1L×T3D intertypic reassortant viruses (originally generated as described (Drayna and Fields, 1982; Brown *et al.,* 1983)) are laboratory stocks. Viruses were grown in mouse L929 cell monolayers in Joklik modified minimal essential medium (GIBCO, Grand Island, NY) supplemented to contain 2.5% fetal calf serum (Intergen, Purchase, NY), 2.5% VSP neonate bovine serum (Biocell, Carson, CA), 2mM glutamine, 100U penicillin per ml, 100μg streptomycin sulfate per ml, and 1μg amphotericin-B per ml, as previously described (Coombs *et al.,* 1994). The identities of strain and reassortant types were confirmed by RNA electropherotyping (Hazelton and Coombs, 1995; Nibert *et al.,* 1996a).

Virus and subviral particle purification

Mouse L929 cells at a concentration of 2×10^7 per ml were inoculated with viral cell lysate stocks at a multiplicity of infection of 5-10 plaque-forming units per cell as described (Coombs *et al.,* 1990). Cells were diluted to a concentration of 6×10^5 per ml in spinner cultures and grown for 66h at 34°C. Where necessary, virus proteins were labeled by adding either 25μCi/ml of Tran^{35}S (ICN Biomedical, Costa Mesa, CA), or 50μCi/ml of ^{3}H-amino acids mixture (ICN Biomedical) to infected cultures at 0 hr postinfection. Infected cells were harvested by low-speed centrifugation, and resuspended in homogenization buffer (250mM NaCl, 10mM β-mercaptoethanol, 10mM Tris, pH 7.4) supplemented to contain 0.1U/ml aprotinin, 40μg/ml phenylmethylsulfonyl fluoride, and 0.5 μg/ml each of tosyl-l-lysine chloromethyl ketone, leupep-

tin, and pepstatin A. Virus-infected cells were freeze-thawed and Freon-extracted twice, as previously described (Smith *et al.,* 1969; Furlong *et al.,* 1988). The final aqueous phases were layered onto 1.25-1.45g/ml cesium chloride gradients and virus was banded at 23,000rpm for at least 4 hours in an SW28 type rotor. Virus bands were dialyzed extensively against dialysis buffer (150mM NaCl, 15mM $MgCl_2$, 10mM Tris, pH 7.4). Virus concentration was measured by optical density at 260nm, using the relationship 1 ODU $= 2.1 \times 10^{12}$ particles per milliliter (Smith *et al.,* 1969). Intermediate Subviral Particles (ISVPs) and cores were generated by digesting appropriate concentrations of the dialyzed, purified virus with 200μg/ml of TLCK-treated chymotrypsin at 37°C, as described (Joklik, 1972; Nibert *et al.,* 1991). The reactions were stopped, at 1 hr of digestion for ISVPs or 3 hr of digestion for cores, by the addition of PMSF to 2.5mM and chilling on ice. All subsequent purification steps were performed at 4°C to prevent conversion of the purified ISVPs to cores. Virions, ISVPs, and cores then were doubly gradient-purified. Particles were initially purified by rate-zonal centrifugation in 10-40% sucrose gradients (Beckman SW28 rotor; 25,000rpm; 60min for virus and ISVP, and 90min for cores). Bands were collected, layered onto 1.22-1.55g/ml cesium chloride gradients and particles re-banded at 26,000rpm for $\geq$ 6 hr. Virions and ISVP samples were dialyzed against dialysis buffer. Core samples were dialyzed against Core Buffer (1M NaCl, 100mM $MgCl_2$, 25mM HEPES, pH 8.0). The identities of each type of particle were confirmed by electron microscopy and protein profile in SDS-PAGE.

SDS-PAGE

Viral particles were dissolved in electrophoresis sample buffer (0.24M Tris, pH6.8, 1.5% dithiothreitol, 1% SDS). Samples were heated to 95°C for 3-5 minutes, and proteins were resolved in either 5-10% linear gradient SDS-PAGE in the phosphate-urea buffer system (Summers *et al.,* 1965), or in 4-16% linear gradient SDS-PAGE using the discontinuous tris-glycine buffer system (Laemmli, 1970) (16.0 $\times$ 18.0 $\times$ 0.1cm). Simultaneous resolution of viral RNA, proteins, and major cleavage peptides was performed in 4-16% exponential gradient Laemmli SDS-PAGE. Components of the phosphate-urea and tris-glycine gels were used to generate a hybrid resolving tris-glycine-urea (TGU) gel that was found to resolve all virus structural proteins (detailed earlier). This hybrid resolving gel used the tris-glycine SDS-PAGE buffer system and consisted of a 4cm step of 4% acrylamide (which contained no urea) on top of a 14 cm tall exponential gradient of 4-16% acrylamide that was supplemented with 7M urea. After electrophoresis slab gels were fixed in 30% isopropanol, 10% acetic acid and then either fluorographed (Enlightning, Dupont), or silver-stained (Merril *et al.,* 1981) and/or Coomassie-stained. Peptide analyses of viral proteins after tryptic, or *S. aureus* V8 protease, digestion was carried out as described (Cleveland *et al.,* 1977). Fluorographs of films of resolved viral proteins were scanned with an LKB integrating laser densitometer. Band intensities were determined by linear regression analysis of multiple exposures to locate the linear response of the film. Radioactive decay of some resolved proteins was measured with a phosphorimager (Molecular Dynamics PhosphorImager SF). Viral gene segments were resolved in 10% SDS-PAGE (16.0 $\times$ 17.5 $\times$ 0.15cm), stained with ethidium bromide, visualized on an ultraviolet light box, and photographed with a Polaroid camera.

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