Mutational Analysis of the Capsid Protein of *Leishmania* RNA Virus LRV1-4

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The virion of *Leishmania* RNA virus is predicted to be composed of a 742-amino-acid major capsid protein and a small percentage of capsid-polymerase fusion molecules. Recently, the capsid protein alone was expressed and shown to spontaneously assemble into viruslike particles. Since the major structural protein of the virion shell self-assembles into viruslike particles when expressed in the baculovirus expression system, assembly of the virion can be studied by mutational analysis and expression of a single open reading frame. In this study, several deletions and one addition of the capsid protein of *Leishmania* RNA virus LRV1-4 were generated. These mutants show different degrees of assembly. Assembly domains are being identified such that the capsid protein may be used as a macromolecular packaging and delivery system for *Leishmania* species.

Viruses of the protozoan parasite *Leishmania* species were originally sought both for their potential in assisting the understanding of the molecular mechanisms of the host organism and as a possible gene transfer system, which, in these organisms, has only recently become available (3, 15). While electroporation of DNA into *Leishmania* species has become common practice in the last few years, there is currently no system available for RNA transfer into these parasites.

Viruses were first discovered in protozoan parasites in 1960 (16) but were not found in *Leishmania* species until 1988 (23, 28). *Leishmanivirus* (LRV1) is a double-stranded RNA virus which appears to have a coding and replicative strategy similar to that of other double-stranded RNA viruses of simple eukaryotes (11, 27). For this reason, *Leishmanivirus* has recently been classified as a member of the *Totiviridae* family of viruses (17). Twelve isolates of LRV1 have been identified and are designated LRV1-1 through LRV1-12 (17).

The sequence of cDNA copies of viral genomic RNA (20, 21) reveals a coding capacity for at least two proteins. No function has yet been determined for the small 5' open reading frames (ORFs) (Fig. 1A). ORF2 could encode an 82-kDa protein, and ORF3 could encode a 98-kDa protein. The virus appears to be regulated and maintained at a low copy number within infected cells (7, 8), making detection of the proteins difficult. Western blot (immunoblot) analysis revealed a major viral protein of about 80 kDa (5), the size predicted from sequence information to be encoded by ORF2 (20, 21). This protein has been shown by *in vitro* translation (5) and expression in the baculovirus expression system (6) to be encoded by ORF2. Self-assembly of recombinant capsid proteins expressed in insect cells, using the baculovirus expression system, has been observed for several other viruses (10, 14, 22). ORF2, when expressed in a baculovirus expression system, self-assembles into viruslike particles (6) in the cytoplasm of recombinant baculovirus infected *Spodoptera frugiperda* (SF9) cells, indicating that ORF2 does encode the capsid protein.

The predicted amino acid sequence of ORF3 has motifs characteristic of viral RNA-dependent RNA polymerases (4) and is therefore predicted to encode the viral polymerase. This ORF overlaps the end of ORF2 by 71 nucleotides. Sequence analysis of the ORF2/ORF3 overlap indicates that this region could form a pseudoknot structure similar to those of retroviruses, suggesting a translational frameshift to produce a Gag-Pol-type fusion protein (21), as is seen in both yeast L-A virus (9, 13) and *Giardiavirus* (26). This fusion protein would have the polymerase domain attached to the C terminus of the capsid protein.

In this study, deletion mutants of the capsid protein were expressed by using the baculovirus expression system, and the level of assembly was determined. Identification of conserved regions of the capsid protein was inhibited by the lack of homology to capsid proteins outside the genus LRV1. Unfortunately, the two members of the genus LRV1 which have been completely sequenced, LRV1-1 and LRV1-4 (20, 21), reveal over 90% amino acid identity (96% similarity), the amino acid differences being distributed throughout the capsid protein, with no distinct clustering. In contrast, other members of the family *Totiviridae*, *Giardiavirus* and yeast L-A virus, show less than 20% identity with the capsid region of LRV1-4, with no distinct conserved domains. Limited sequence information available from the recently identified *Leishmanivirus* LRV2-1 (19) reveals a more intermediate degree of homology with LRV1-4, but no distinct conserved regions have been identified within the putative capsid ORF. Without conserved regions to target for mutational analysis, the first mutants constructed were large and small terminal deletions and small internal deletions. These mutants show various degrees of assembly and have provided clues into the mechanism of LRV1-4 virus assembly.

Studies involving the capsid protein may ultimately lead to an RNA packaging and delivery system for *Leishmania* species. It has been shown previously that whole virus particles can be introduced into infected and uninfected *Leishmania* cells (1) by electroporation and infection, respectively. These results indicate LRV1 particles are able to be taken up by uninfected but not infected *Leishmania* cells. While the virus introduced by these methods does not establish a permanent infection, electroporation or direct infection could be used as a delivery system of encapsidated macromolecules.

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MATERIALS AND METHODS

Construction of the mutant transfer vectors. The deletion mutants were generated by using different methods. In brief, deletion mutants N2, N3, C2, and C3 were generated by using PCR and the oligonucleotides listed in Table 1. Plasmid pTCB2 was used as the template; restriction enzyme recognition sites in the amplification primers were used for subsequent cloning into the transfer vector pVL1392 in each case. The transfer vector pTCB2 (6) was originally generated by reverse transcription and PCR amplification of ORF2 from viral RNA, using primers containing *NheI* restriction sites at the termini. These fragments were cloned into the *XbaI* site of pVL1392. Mutants I1 and I2 were generated by BAL 31 digestion of the vector pTCB2 at unique restriction sites within the capsid ORF. Deletion mutants N1 and C1 were generated by subcloning a restriction fragment of the capsid ORF (ORF2) into transfer vector pVL1392.

Specifically, deletion mutant N1 was generated by digestion of pTCB2 with the restriction enzymes *PstI* and *BamHI* and ligation of this fragment into the same sites in pVL1392 (Fig. 1). Deletion mutant C1 was generated by digestion of pTCB2 with the restriction endonuclease *KpnI* and religation of the gel-purified plasmid.

Deletion mutants I1 and I2 were generated by digestion of ORF2 with *HindIII* and *EcoRV*, respectively, and subsequent digestion with BAL 31 slow enzyme. Since the capsid ORF has few unique restriction endonuclease recognition sites and the vector also contained these restrictions sites, it was necessary to transfer ORF2 into another plasmid to eliminate these sites prior to BAL 31 treatment. ORF2 was removed from transfer vector pTCB1 (6) by digestion with restriction enzyme *NheI* and gel purification (see below) of the 2.2-kb fragment containing all of ORF2. This fragment was ligated into the *XbaI* restriction site of vector pBSKS+ (Stratagene). The resulting plasmid was digested with *EcoRI* and *EagI*, and the fragment containing all of ORF2, and the additional restriction endonuclease recognition sequences, was gel purified. This insert was ligated into the *EcoRI* and *EagI* sites of the plasmid pBR322 (New England Biolabs). This new plasmid (pKV) was digested with either *HindIII* or *EcoRV* and subjected to BAL 31 digestion. Ten micrograms of linearized plasmid DNA was digested with 1.6 U of BAL 31 slow enzyme (Kodak) for 21 min at 37°C in the reaction buffer supplied by the manufacturer according to the manufacturer's instructions. The reaction was stopped by addition of EGTA (final concentration of 20 mM), and the enzyme was heat inactivated at 65°C for 5 min. The BAL 31-treated plasmids were self-ligated and sequenced for in-frame deletions. The two mutants that were selected (Fig. 1) contained deletions from bases 676 to 726 (relative to the start point), which was designated I1, and 1312 to 1329, which was designated I2. These mutant plasmids were digested with restriction endonucleases *EcoRI* and *EagI*, and the fragment containing the mutant ORF2 was ligated into pBSKS+. Finally, these plasmids were digested with *PstI* and *NotI*, and the fragment containing the mutant ORF2 was inserted into the *PstI* and *NotI* sites of pVL1392.

Agarose gel purification. In each case, prior to ligation, plasmids and inserts were purified by agarose gel electrophoresis, ethidium bromide staining, and removal of the band of interest. This gel slice was then crushed with an Eppendorf pestle and soaked in TE (10 mM Trizma base [pH 8.0], 1 mM EDTA), and the supernatant was separated from the agarose by centrifugation through a Spin-X filter unit (Costar).

Construction of recombinant baculovirus. The generated transfer vectors (3 µg) were transfected into *S. frugiperda* (SF9)

TABLE 1. Oligonucleotides used for the construction of capsid deletion mutants N2, N3, C2, and C3

Mutant	Sequence ^a		Sites used in pVL1392
	Forward sense primer	Reverse sense primer	
N2	(NA24) 5'-GAAGATCTATCATGAACAGAGAGAGGTTGATC	(BAC32) 5'-TGGATGTCAGCCACTAATCATACGTCG	<i>BglII</i> <i>XbaI</i>
N3	(5A6) 5'-GAAGATCTATCATGATGAAGATTGCTTGGC	(BAC32) 5'-TGGATGTCAGCCACTAATCATACGTCG	<i>BglII</i> <i>XbaI</i>
C2	(BAC32) 5'-GCTGTGCTAGCATGGCTGATATACCAAC	(CA24) 5'-CGCGGATCTCATCATTAAGAAGATGTACACG	<i>XbaI</i> <i>BamHI</i>
C3	(BAC32) 5'-GCTGTGCTAGCATGGCTGATATACCAAC	(CRB+) 5'-CGCGGATCTCTCAACATTGGGAGCGACCGGATCAGCTCGCCGAGCGGACCTCGTTGTC	<i>XbaI</i> <i>BamHI</i>

^a Primer restriction sites are shown in boldface; name designations of the primers are in parentheses.

FIG. 1. Cloning strategy for deletion mutants. (A) Schematic representation of the genome organization of LRV1-4. (B) Map of the transfer plasmid (pVL1392) used for deletion mutants, including restriction endonuclease recognition sites within the multiple cloning region (MCR). (C) Map of the LRV1-4 capsid ORF (ORF2), including the restriction endonuclease recognition sequences used for subcloning of deletion mutants. The asterisk indicates the predicted start site for deletion mutant N1. (D) Map of the deletion mutants. The black boxes indicate regions of the plasmid deleted in the mutants (approximately to scale). The box in C3 indicates the region added to the C3 mutant. The numbers in parentheses indicate the amino acids deleted in each mutant.

cells along with 1 μ g of linear baculovirus (*Autographa californica* nuclear polyhedrosis virus [AcMNPV]) DNA, using cationic liposomes according to the manufacturer's instructions (Linear AcNPV DNA Transfection Module; Invitrogen). Recombinant plaques were identified visually, by the absence of occlusion bodies, and plaque purified (22). Expression of mutant capsid protein was assessed by cross-reaction in Western blot analysis to antiserum generated to LRV1-4 virus purified from M4147 cells (5).

Western blot analysis and Coomassie blue staining of proteins. To determine mutant protein expression levels, 10^6 SF9 cells were infected in a 12-well tissue culture dish, at a high multiplicity of infection, with high-titer recombinant baculovirus stocks. Cells were harvested at day 3 postinfection by lysing in 100 μ l of 1 $\times$ Laemmli sample buffer. The lysate was denatured at 100°C for 5 min and vortexed vigorously (to shear DNA in the sample). A portion of this sample, 4 μ l for Western blot analysis and 10 μ l for Coomassie blue staining, was electrophoresed on a sodium dodecyl sulfate (SDS)-11% polyacrylamide gel. Protein samples from sucrose gradients (5 μ l) were mixed with an equal volume of 2 $\times$ Laemmli sample buffer, denatured at 100°C for 5 min, and electrophoresed on SDS-9% polyacrylamide gels for Western blot analysis.

Western blot analysis was performed by transferring the proteins from the SDS-polyacrylamide gel to a polyvinylidene difluoride membrane (Millipore Immobilon-P) with a Hoefer Semi-Phor electrotransfer unit. The blot was blocked overnight at 4°C in phosphate-buffered saline with 0.05% Tween 20 (TPBS) plus 5% Carnation nonfat dry milk. The blot was incubated in a 1/2,000 dilution of precleared anti-LRV1-4 antiserum (5) in TPBS plus 5% milk for 90 min at room temperature and washed for 30 min in TPBS at room temperature, with at least five changes of wash. The blot was then incubated in a 1/5,000 dilution of affinity-purified goat anti-

rabbit immunoglobulin G (Kirkegaard & Perry) in TPBS plus 5% milk for 1 h at room temperature and washed as before. The alkaline phosphatase reaction was performed as instructed by the manufacturer (Gibco BRL). Note that the Western blot of the C3 sucrose gradient shown in Fig. 3 contains four times the amount of protein as the other gradients, and the sample was sonicated prior to loading onto the sucrose gradient. This procedure was used to help separate aggregates of assembled mutant capsid and to obtain a clearer signal in fraction 4 for publication. Prior to sonication, with amounts of protein loaded equivalent to the other gradients, there was a very faint band observed in fraction 4 on Western blots (not shown).

Coomassie blue staining of gels was performed by soaking the gels in a 0.25% solution of Coomassie brilliant blue R250 in 45% methanol-10% glacial acetic acid for 2 h and destaining in 45% methanol-10% glacial acetic acid for several hours.

Sucrose gradient sedimentation of expressed viruslike particles. Viruslike particles were generated by infecting a 60-mm-diameter tissue culture dish containing 2×10^6 SF9 cells with recombinant baculovirus at a multiplicity of infection of 10. The infected cells were harvested at day 3 by scraping the cells from the plate in 0.5 ml of lysis solution (50 mM Trizma base [pH 8.0], 150 mM NaCl, 2% Nonidet P-40), loaded onto an 11-ml, 10 to 40% sucrose in HCN buffer (50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [HEPES; pH 7.4], 5 mM CaCl₂, 150 mM NaCl) gradient, and centrifuged at 36,000 rpm in a Beckman SW41 rotor for 135 min at 4°C. Sucrose gradients were collected in 1-ml fractions and numbered from the bottom of the gradient. The pellet of each gradient was resuspended in 1-ml gradient buffer (HCN).

EM. Sucrose gradient-purified particles were allowed to adhere to a carbon film, stained with 1% uranyl formate, and lifted onto copper electron microscopy (EM) grids. Negative staining was done by a miniaturization of the method of

TABLE 2. Summary of assembly of the mutant capsid proteins as determined from sedimentation analysis and EM

Mutant	Protein in fraction 6 ^a	Particles seen by EM	Assembly
Full length	++++	++++	+
N1	0	0	—
N2	0	+	—/+
N3	++++	++++	+
I1	0	0	—
I2	0	0	—
C1	+++	++	+
C2	+++	+++	+
C3	+	++	+

^a Relative amount of cross-reacting protein which is present in fraction 6 of a 10 to 40% sucrose velocity gradient.

Valentine and Green (25). It was found that the best method for visualization of the viruslike particles was to lift the particles directly from the sucrose gradient fractions onto carbon and float the carbon on stain for 2 min as described. Two minutes of floating on stain was necessary to remove sucrose and to exchange the ions in the buffer for stain. Removal of the sucrose by floating the carbon on water prior to staining did not aid visualization, and staining for less than 2 min resulted in very poor visualization and some caramelization of the remaining sucrose. Dialysis of the viruslike particles into low or even moderate ionic strength, similar to the gradient buffer itself, tended to aggregate the viruslike particles into clumps too large to be penetrable to the electron beam.

RESULTS

Without even moderate sequence homology to other capsid proteins, it was difficult to predict what regions of the capsid may be important for assembly. The first mutants constructed were therefore large and small terminal deletions and small internal deletions, as shown in Fig. 1. Initial examination of assembly involved an analysis of sedimentation through sucrose gradients. The capsid mutants were then further analyzed by EM. The construction of the mutants is outlined in Table 1 and in Materials and Methods; data on assembly are summarized in Table 2. It has been shown previously that expression of ORF2 in insect cells by using the baculovirus expression system produced a protein which self-assembles into viruslike particles in the cytoplasm of infected cells (6). It was also demonstrated that a specific sedimentation pattern through a sucrose gradient correlated with the assembly into viruslike particles. Under the conditions defined in Materials and Methods, the baculovirus-expressed particles sediment with a peak of approximately fraction 6 in a sucrose gradient. Native LRV1-4 particles sediment somewhat lower in the gradient, with a peak of approximately fraction 4. Unassembled proteins remain at the top of the gradient or are in the pellet of the gradient. The mutant capsid proteins were therefore analyzed for their sedimentation properties through sucrose gradients, as a first indication of level of assembly.

Several fractions of the sucrose gradient were examined by EM. The majority of total proteins, as determined from Coomassie blue staining, were at the top of the gradient, or in the pellet of the gradient, including baculovirus particles as seen by EM. However, even when no protein was visible in the gradient fraction by Coomassie blue staining or Western blot analysis, in most cases there were some protein structures, which may be substructures of LRV1-4 particles, in fraction 6. Fraction 6 is where the recombinant particles are most abun-

FIG. 2. Expression of mutant ORF2 in *S. frugiperda* (SF9) cells, demonstrated by Coomassie blue staining (A) and Western blot analysis (B) of proteins from lysates of 10^5 SF9 cells mock infected (lanes 2), infected with wild-type AcMNPV (lanes 3), or infected with recombinant AcMNPV expressing full-length LRV1-4 capsid protein (lanes 4), deletion mutant N1 (lanes 5), deletion mutant N2 (lanes 6), deletion mutant N3 (lanes 7), deletion mutant I1 (lanes 8), deletion mutant I2 (lanes 9), deletion mutant C1 (lanes 10), deletion mutant C2 (lanes 11), and addition mutant C3 (lanes 12). SF9 cells were infected with the indicated virus at a multiplicity of infection of 10 and harvested at day 4 postinfection. Lanes 1, protein molecular weight markers. Sizes are indicated in kilodaltons.

dant when the full-length capsid is expressed. These structures (see Fig. 4) were found only in infected cells expressing recombinant capsid protein, not in cells infected with wild-type baculovirus. The number of these particles correlated to the amount of mutant capsid protein, as determined by Western blot analysis.

Amino-terminal capsid mutants. The transfer vector containing the large amino-terminal truncation (N1) was cloned and expressed as described in Materials and Methods. The mutant capsid protein was expressed at a lower level than the wild-type capsid protein, as seen by Coomassie blue staining and Western blot analysis (Fig. 2). The lower level of expression was presumably due to the longer 5' untranslated region included in the cloning process and the necessity for the capsid to initiate at an internal methionine (Fig. 1C, asterisk). The recombinant virus did produce large enough quantities of mutant protein for further analysis, and the protein produced electrophoresed as a single band of approximately the expected size (about 56 kDa), indicating the likelihood that the internal initiation probably occurred at the predicted location in the sequence. The majority of the immunocross-reacting protein produced from this mutant was found in the pellet of the sucrose gradient (Fig. 3, N1), indicating that the protein did not assemble. This inference is supported by the lack of viruslike particles observed by EM (Fig. 4, N1).

The small amino-terminal mutants N2 and N3 were generated by PCR as described in Materials and Methods and Table 1. The initiating methionine was retained in the same context as the native virus, both in an attempt to retain similar levels of expression from the expressed protein and because the amino-terminal methionine was found to be essential for assembly of the yeast L-A virus (24). Both mutants expressed abundant immuno-cross-reactive protein similar in size to the full-length capsid protein (Fig. 2). The smallest mutant, N3, which was missing only six amino acids, was still fully competent for assembly, as determined by sedimentation analysis and EM (Fig. 3 and 4). The larger mutant, N2, although it was missing only 24 amino acids, was not able to assemble efficiently (Fig. 3 and 4).

Internal deletion capsid mutants. The two internal deletion mutants, I1 and I2, were missing 17 and 7 amino acids, respectively. Recombinant virus expressing I1 and I2 both produced an immuno-cross-reactive protein that was similar in size to the full-length capsid (Fig. 2). However, both were defective for assembly. This lack of assembly is due to the loss of residues critical for either intermolecular interactions involved in assembly of capsomers or intramolecular interaction involved in proper folding of the capsid protein. While it is not possible to delineate the differences between these possibilities, it is interesting that two such small internal deletions are so deleterious.

Carboxy-terminal capsid mutants. Interestingly, both the large and small carboxy-terminal deletion mutants, C1 and C2, respectively, retain the ability to assemble, as determined by sedimentation and EM analysis (Fig. 3 and 4). The small C-terminal deletion mutant is missing the last 24 amino acids of the capsid protein. These amino acids are not predicted to be present in the capsid-polymerase fusion protein, since they are downstream of the predicted frameshift (see below).

The RNA binding domain of hepatitis B virus was added to the C terminus of the capsid protein. This small C-terminal addition mutant, C3, was also determined by EM to assemble. Initial sucrose gradient analysis indicated that the majority of the C3 mutant capsid protein was in the pellet of the gradient, much like the mutants which do not assemble. However, EM analysis indicated that unlike the case for other mutants, the pellet contained large aggregates of viruslike particles, many of which appear not fully closed, or coiled. The reason for the aggregation is unclear. One explanation is that the added amino acids are exposed on the surface of the capsid and interfere with the surface charge, causing aggregation. However, the added residues, according to our model for viral replication, are predicted to be internal to the particle. While the aggregation of particles does not support this theory, it is possible that the additional amino acids, which constitute a nucleic acid binding domain for hepatitis B virus, are binding nucleic acids and therefore not allowing the particle to fully close. Binding nucleic acids could cause aggregation if more than one viruslike particle bound the same molecule of nucleic acid. The C3 particles have unusual structures internal to the particle (Fig. 4, C3). While it is difficult to interpret the meaning of these structures, if they are derived from the additional amino acids from hepatitis virus, then the carboxy

terminus of the capsid must be internal to the particle, which is supportive of the replication model, as discussed below. This addition mutant appears to yield conflicting data as to the location of the C terminus of the protein. However, if the asymmetric subunit of the capsomer is a dimer of the capsid protein, as is the case in the yeast L-A virus (2), the C terminus could be both internal and external to the particle.

DISCUSSION

Not surprisingly, the large amino-terminal deletion, N1, was assembly incompetent. Interestingly, the protein of this mutant, when dialyzed, tended to aggregate in a specific manner, forming what appeared to be partially assembled capsids (Fig. 4, N1d), while the directly stained proteins remained dispersed (Fig. 4, N1). This finding indicates that without the salt to mask the charged residues, some of the contacts which are likely involved in assembly of the particle can still be generated.

The other amino-terminal mutants provide significant information about assembly. The smallest deletion mutant, N3, which was missing six of the seven amino-terminal amino acids, was capable of assembly. The other small deletion mutant, N2, which was missing only 24 amino acids, showed a diminished ability to assemble. Those few particles that were observed in the N2 mutant appeared very unstable (Fig. 4). This finding indicated that the amino terminus is important for assembly, but deletion of a few amino acids is not sufficient to abolish assembly. It is also of note that the amino-terminal residues are more critical for assembly than the carboxy-terminal residues.

The two internal deletion mutants were both diminished in ability to assemble. This could be due either to the elimination of residues directly involved in contacts important for assembly or to the disruption of structure of a larger region of the capsid protein, which is more likely to occur with internal deletions than terminal deletions.

Interestingly, the carboxy-terminal deletion mutants were competent for assembly. This finding has important implications for the predicted replicative strategy of the virus, which predicts that the polymerase is included in the capsid directly by assembly of the capsid domain of the capsid-polymerase fusion protein into the virion shell. Since the 24 amino acids deleted in mutant C2 were not predicted to exist in the fusion protein, and mutant C2 is still competent to assemble, it is at least possible for the predicted fusion protein to assemble as part of the virion shell as predicted.

It was surprising to us that the large C-terminal deletion mutant was still able to assemble. This finding has important implications for future manipulations of the capsid, since this region is predicted to be internal to the particle. This result indicates that it may be possible to delete a large region of the carboxy terminus of the protein and retain assembly, such that a larger amount of space inside the particle may be made available for other purposes.

The hepatitis B virus RNA binding domain was selected for addition to the capsid protein (C3 mutant), since it was defined as a small (eight amino acids) region and had previously been shown to bind heterologous RNAs (12). To provide a success-

FIG. 3. Western blot analysis across fractions from a 10 to 40% sucrose gradient of recombinant baculovirus-infected SF9 cells. Gradient fractions are numbered 1 through 11 from the bottom of the gradient, and the same fractions are shown left to right. P, pellet of the gradient; +, expressed capsid protein control. Molecular masses are indicated in kilodaltons. Western blots were generated from SF9 cells infected with recombinant AcMNPV expressing full-length capsid protein (C), deletion mutant N1, deletion mutant N2, deletion mutant N3, deletion mutant I1, deletion mutant I2, deletion mutant C1, deletion mutant C2, and addition mutant C3 (note different processing of C3 described in Materials and Methods).

FIG. 4. Electron micrographs of wild-type (wt) virus particles (from *Leishmania* sp. strain MHOM/BR/75/M4147), full-length baculovirus-expressed capsid protein (C), deletion mutant N1, deletion mutant N1 after dialysis (N1d), deletion mutant N2, deletion mutant N3, deletion mutant I1, deletion mutant I2, deletion mutant C1, deletion mutant C2, and addition mutant C3. Magnification, $\times 115,000$.

ful RNA packaging and encapsidation system, this region must be expressed internally to the assembled particles and bind heterologous RNAs. While this domain is expressed and particles still retain the ability to assemble, RNA has yet to be demonstrated within the particles, and RNA binding ability has not been established with the expressed C3 mutant protein. While further manipulations will be required to develop an RNA packaging system, the C3 mutant did provide us with valuable clues about the structure of the capsid. The presence of internal structures upon addition to the C terminus indicates that the added residues are likely to be internal to the viruslike particle, which is supportive of the predicted replicative model (11). Also, while the sequence of the C3 mutant predicted a protein indistinguishable in size from the full-length capsid, the C3 mutant electrophoresed anomalously large. When purified by sucrose gradient, the mutant capsid produced a doublet similar to what has been seen with the full-length baculovirus-expressed capsid as well as the native LRV1-4 particles. Interestingly, while the expressed protein appears anomalously large, the faster-migrating species of C3 is the same size as the faster-migrating species produced from the wild-type capsid. This finding, while not conclusive, supports the notion that the capsid is cleaved at the C terminus.

The ultimate goal of this work is to devise an RNA packaging and delivery system for *Leishmania* species. At this time, transfer of DNA via electroporation is commonly in use, but there has never been a successful transfer of RNA into *Leishmania* cells. This inability could be due to the rigors of electroporation and the relative instability of RNA. Transformation via electroporation of DNA into *Leishmania* cells also requires large quantities of DNA. Typical transformation protocols yield about two transformants per microgram of DNA and require weeks for the transformant to outgrow untransformed cells in culture, further diminishing the likelihood that direct electroporation could be used for a transient RNA based transfection. Electroporation of whole LRV1 (1) virus particles into heterologously infected *Leishmania* cells gave a transient infection that did not persist. Infection of uninfected *Leishmania* cells also resulted in a transient infection which persisted longer than the heterologous infection of infected cells but did not require electroporation. These results suggest that the capsid protein may be used for the transfer of macromolecules into *Leishmania* cells.

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