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# Structural Organization of Poliovirus RNA Replication Is Mediated by Viral Proteins of the P2 Genomic Region

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**Transcriptionally active replication complexes bound to smooth membrane vesicles were isolated from poliovirus-infected cells. In electron microscopic, negatively stained preparations, the replication complex appeared as an irregularly shaped, oblong structure attached to several virus-induced vesicles of a rosettelike arrangement. Electron microscopic immunocytochemistry of such preparations demonstrated that the poliovirus replication complex contains the proteins coded by the P2 genomic region (P2 proteins) in a membrane-associated form. In addition, the P2 proteins are also associated with viral RNA, and they can be cross-linked to viral RNA by UV irradiation. Guanidine hydrochloride prevented the P2 proteins from becoming membrane bound but did not change their association with viral RNA. The findings allow the conclusion that the protein 2C or 2C-containing precursor(s) is responsible for the attachment of the viral RNA to the vesicular membrane and for the spatial organization of the replication complex necessary for its proper functioning in viral transcription. A model for the structure of the viral replication complex and for the function of the 2C-containing P2 protein(s) and the vesicular membranes is proposed.**

Polioviral plus-strand RNA is synthesized in the partially double-stranded (35) replicative intermediate which, from biochemical work, was postulated to be confined in a membrane-bound structure called the replication complex (7, 8, 15, 19; for a review, see reference 21). In a recent ultrastructural and high-resolution autoradiography study (2), the viral replication complex was visualized and demonstrated to be formed on membrane protrusions at the rER. These protrusions grow into virus-induced vesicles characteristic of picornavirus-infected cells (5, 10, 13). The replication complex remains associated with the outer surfaces of these vesicles (2). Immunocytochemical investigations, using monoclonal antibodies against proteins of the poliovirus genomic region P2 (33) (poliovirus protein nomenclature according to reference 37), showed that the P2 proteins 2BC and 2C are contained exclusively within the replication complex and on the vesicular membranes (2).

The role of the viral proteins encoded in the P2 genomic region is not understood entirely. The protein 2A was demonstrated to be a viral proteinase (43), and the protein 2BC was reported (4) to be involved in the induction of the membrane proliferation within the host cell, leading to the vesicles mentioned above. Linker insertion mutants in the 2C-coding genomic region were found to be defective in viral RNA synthesis, and it was concluded that the protein 2C is needed continually for this synthetic activity (24).

Guanidine, in millimolar concentrations, blocks viral RNA synthesis, specifically initiation (9, 30). Guanidine-resistant or -dependent poliovirus mutants have been mapped to the genomic region of the viral protein 2C (34). Therefore, any viral function which is guanidine sensitive can be considered dependent on or exerted by the viral protein 2C (or, possibly, a 2C-containing precursor peptide). This is in accord with the findings (24) that the protein 2C is involved in viral RNA synthesis.

In addition to the protein 2C, the virus-induced vesicles (or, presumably, their membranes) were shown to be a prerequisite for viral RNA synthesis (3, 11, 40). In the present investigation, therefore, we examined the interaction of the protein 2C with its counterparts, i.e., the viral replication complex, the vesicular membranes, and the viral RNA. Using electron microscopy (EM) and EM immunocytochemistry (IEM) of isolated virus-induced vesicles, we could demonstrate the size and shape of the viral replication complex, its spatial relation to the vesicular membrane, and the location of the P2 proteins in the functional, membrane-bound replication complex. To test a possible association of the P2 proteins with viral RNA, we performed UV photocross-linking experiments in infected, intact cells during the period of peak viral RNA synthesis. We found a small amount of the P2 proteins to be in close contact with the replicating viral RNA, so that UV light could induce covalent bonds between the two components (20). The association of the P2 proteins with RNA was not changed after guanidine-induced blockage of viral RNA synthesis, but the attachment of the P2 proteins to the vesicular membranes was abolished by the drug. This, in turn, prevents the association of the replication complex as a whole with the vesicular membranes. Our conclusion is that the protein 2C, and perhaps its precursor(s), attaches the viral RNA to the vesicular membranes, thereby providing the correct spatial organization of the RNA necessary for its replication.

## MATERIALS AND METHODS

**Cells and virus.** HEp-2 cells and poliovirus type 1 (Mahoney) were grown in suspension cultures as described elsewhere (5). The multiplicity of infection for the experiments was 30 PFU per cell.

**Labeling of viral proteins and viral RNA.** Viral proteins were labeled by pulsing the infected cells with 75 μCi of L-[4,5-$^3$H]leucine (Amersham International, Amersham, United Kingdom) per ml in the presence of 120 mM excess NaCl (31) to suppress any residual cellular protein synthesis. To label viral RNA, cells were treated with 5 μg of dactinomycin (Merck Sharp & Dohme, Rahway, N.J.) per ml 30 min prior to the addition of 25 to 200 μCi of [5-$^3$H]uridine

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(Amersham) per ml. Cytoplasmic extracts were prepared by Dounce homogenization and low-speed centrifugation as described elsewhere (4).

**Guanidine treatment.** Guanidine hydrochloride (final concentration, 2 mM) (Fluka, Buchs, Switzerland) was added to the infected cells at 2.75 or 3 h postinfection (p.i.). This treatment reduced viral RNA synthesis in approximately 10 min to background levels of less than 5% of an untreated, infected culture. Guanidine is very easily washed out from the cells (29; our own observations with [$^{14}$C]labeled guanidine). Therefore, we found it necessary to include the drug in all solutions (e.g., buffers, sucrose gradient, immunochemical reagents, etc.) used to process the subcellular fractions in order to avoid any rearrangement of the vesicles and the replication complex during isolation and immunocytochemical preparations. The time p.i. of the guanidine addition was chosen to obtain virus-induced cell alterations (i.e., vesicle formation and choline incorporation [3]) comparable to that in infected cells not treated with guanidine. If guanidine was added earlier, the virus-induced vesicle formation did not occur or did not reach the same extent as that in an infected, untreated control.

**MAb.** Monoclonal antibodies (MAb) against proteins of the P2 genomic region of the poliovirus were characterized previously (2, 33). They recognize an epitope in the C-terminal part of the P2 region, i.e., in protein 2C and, consequently, in its precursors 2BC and P2. They will be referred to as 2C-MAb. The MAb against poliovirus capsid proteins were prepared in the same way as the 2C-MAb, using the appropriate proteins electroeluted from acrylamide gels as immunizing antigens. In this study, MAb of the clone B3/H.1 was used, recognizing the capsid protein VP1 and its precursors. The hybridoma culture supernatants were used undiluted for Western blots (immunoblots) and diluted 1:2 for immunocytochemistry.

**PAGE, fluorography, and immunoblotting.** Cytoplasmic extracts or sucrose gradient fractions were prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) (22) on 12.5% gels as described elsewhere (3). For fluorography, the gels were impregnated with Enlightning (DuPont, NEN Research Products, Boston, Mass.) and exposed on prefogged Kodak X-Omat film (23). The proteins were quantitated on the fluorographs with a Beckman CDS-200 integrating densitometer. Blotting onto nitrocellulose (Bio-Rad Laboratories, Richmond, Calif.) was done by the method of Towbin et al. (42). Viral proteins were visualized by an immune reaction with MAb and peroxidase-coupled anti-mouse immunoglobulin G as described elsewhere (14).

**UV cross-linking of viral proteins to RNA and isolation of RNP.** To test for RNA-protein interactions, the UV cross-linking method (20) was used. Infected cells were suspended in 10 mM Tris (pH 7.4)–0.9% NaCl–1 mM $MgCl_2$ at a density of $3 \times 10^6$ to $4 \times 10^6$ cells per ml and distributed into petri dishes to obtain fluid layers of about 1-mm thickness. They were irradiated with a Philips TUV germicidal lamp at a distance of 3.5 cm for 20 min at 0°C, which corresponds to a total of $3.4 \times 10^5$ ergs per mm$^2$. In the cytoplasmic extract of the irradiated cells, RNA and not covalently bound protein was dissociated with 4 M guanidinium thiocyanate, and the RNA and ribonucleoprotein (RNP) were isolated by sedimention through cesium chloride (26). To avoid any contamination of the RNA-RNP sediment with free protein, the use of two cesium chloride cushions (density of 1.37 g/cm$^3$ layered on 1.76 g/cm$^3$) was advantageous. For PAGE, the pellet was dissolved in 10 mM Tris (pH 7.4), precipitated with ethanol, dissolved again, and digested with preboiled pancreatic RNase A (25 μg/ml) and micrococcal nuclease (400 U/ml) in 0.5 mM phenylmethylsulfonyl fluoride at 37°C for 30 min or as indicated.

**Filter-binding assay for RNA cross-linked to protein.** To test whether protein was cross-linked to RNA, the pellet from the cesium chloride centrifugation described above was dissolved in 20 μl of TE buffer (10 mM Tris [pH 7.5]–1 mM EDTA) and 2% sodium dodecyl sulfate, diluted to 1 ml with TE buffer and filtered through a 2.5-cm nitrocellulose filter (0.45 μm; type BA85; Schleicher & Schuell, Inc.) (32, 48). Free RNA will go through the filter, but RNP will be retained because of the protein-binding capacity of nitrocellulose. The filter was dissolved in 0.8 ml of ethylenglycolmonoethylether (Cellosolve; Merck), and the radioactivity of the retained viral RNA was determined by liquid scintillation counting.

**Immunoprecipitation.** Immunoprecipitation of RNP was done with 2C- and VP1-MAb bound to a matrix of Sepharose-protein A-rabbit anti-mouse immunoglobulin G (38). Antigen and matrix were incubated at room temperature for 45 min in RIPA buffer (12) in the presence of RNasin (Promega Biotec, Madison, Wis.). The Sepharose was washed several times with RIPA, boiled in Tris buffer containing 4% sodium dodecyl sulfate, centrifuged, and counted in a liquid scintillation counter.

**Isolation and in vitro RNA synthesis of replication complexes attached to virus-induced vesicles.** For the isolation of vesicle-attached replication complexes, the original protocols using discontinuous sucrose gradients centrifuged to equilibrium for 18 h (4, 11) were modified. Cytoplasmic extracts of poliovirus-infected cells were centrifuged onto a double sucrose cushion (0.8 ml of 30% sucrose layered over 0.8 ml of 45% sucrose, both in reticulocyte standard buffer) for 1 h at 300,000 × *g* (average) in an SW55 rotor. The virus-induced vesicles with their replication complexes could be harvested from the top of the 30 and 45% sucrose cushions. Differences between the two fractions will be described in the Results section.

Both the 30 and 45% fractions, when introduced into a cell-free transcription system (40), readily synthesized viral RNA (see Results).

**EM and IEM.** The isolated, vesicle-attached replication complexes were used directly or after in vitro RNA synthesis for EM and IEM. Specimens showing high resolution and good preservation of ultrastructural details were obtained as follows. The vesicle fractions were adsorbed onto nitrocellulose (Parlodion; EMS, Fort Washington, Pa.)-coated EM grids, which were reinforced and rendered hydrophilic by glow discharge in a benzene atmosphere. The immobilized vesicles were immunocytochemically labeled (after nonspecific binding sites were blocked with normal goat serum and bovine serum albumin) by incubating the grids with MAb followed by goat anti-mouse antibodies coupled to colloidal gold of 10 nm (Janssen Pharmaceutica, Beerse, Belgium) as described elsewhere (1). The preparations were negatively stained with 1% phosphotungstic acid, pH 7.0, before being viewed in a Siemens 102 electron microscope operated at 100 or 125 kV. The immunolabeling procedure was found to have no detectable influence on the morphology of the specimens.

## RESULTS

**Association of poliovirus proteins of the P2 genomic region with viral RNA.** RNA-associated proteins were cross-linked to RNA by irradiating infected cells at 3.75 h p.i. with UV light (20). This procedure covalently binds those RNA and

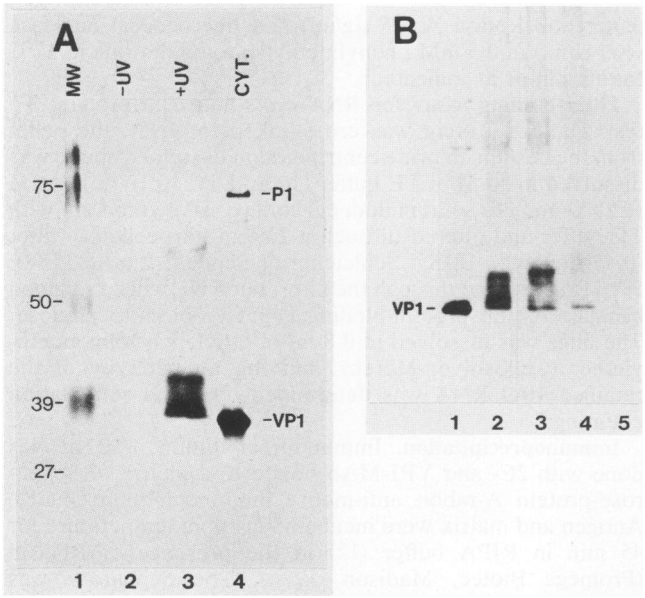



FIG. 1. (A) Immunoblot demonstrating VP1 UV cross-linked to RNA. Poliovirus-infected HEp-2 cells were UV irradiated (+UV, lane 3) or not irradiated (−UV, lane 2) at 3.75 h p.i., and RNP or RNA, respectively, was isolated by the guanidinium-cesium chloride method. The pelleted RNP or RNA was RNase digested, electrophoresed, blotted onto nitrocellulose, and reacted with the VP1-MAb B3/H.1, followed by peroxidase-coupled anti-mouse antibody. CYT. (lane 4), infected cytoplasmic extract not treated with UV light; MW (lane 1), prestained molecular weight standard. (B) Immunoblot reacted with VP1-MAb as described for panel A. Lane 1, Cytoplasmic extract from UV-irradiated cells; lanes 2 to 5, RNP pellets subjected to decreasing RNase digestion before PAGE. Lane 2, 25 μg of RNase A and 400 U of micrococcal nuclease per ml, 37°C, 30 min; lane 3, 2.5 μg of RNase A per ml, 0°C, 2 min; lane 4, 0.25 μg of RNase A per ml, 0°C, 2 min; lane 5, no RNase digestion. RNase treatment is necessary for the viral proteins to enter the gel, and retardation of the electrophoretic mobility of the protein is inversely related to the extent of RNase treatment.

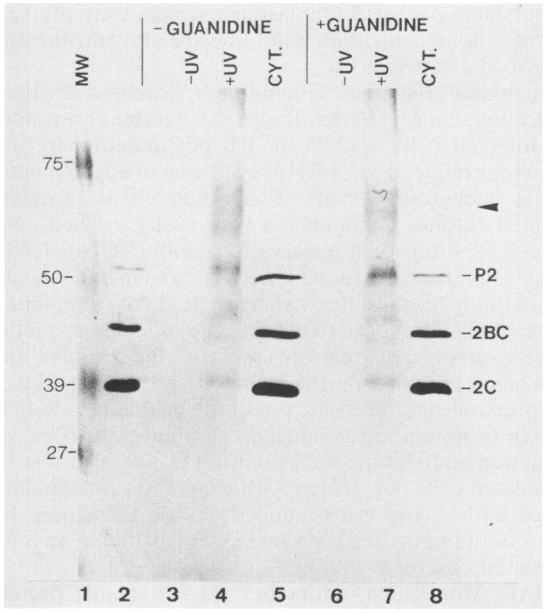



FIG. 2. Immunoblot demonstrating that P2 proteins can be cross-linked to RNA in cells treated and not treated with guanidine. The blot was prepared as described in the legend to Fig. 1A, except that the 2C-MAb 11/2B1.1. was used. Lane 1, Prestained molecular weight (MW) standard; lane 2, our standard cytoplasmic extract of poliovirus-infected cells; lanes 3 to 5, −guanidine, viral RNA synthesis not blocked by guanidine; lanes 6 to 8, +guanidine, 2 mM guanidine added to the culture at 3 h p.i.; lanes 3 and 6, cesium chloride pellet from cells not UV irradiated (−UV); lanes 4 and 7, cesium chloride pellet from UV-irradiated cells (+UV); lanes 4 and 8, respective cytoplasmic extract (CYT.) of the cells not UV irradiated. After UV irradiation, some immunoreactive protein migrates slower than the $P_2$ protein (arrowhead).

protein molecules which, at the time of irradiation, are not farther apart than the distance of one C-C bond (46). Intact cells rather than a cytoplasmic extract were irradiated in order to avoid possible unspecific RNP formation (45) by cross-linking viral RNA and RNA-binding proteins, which are irrelevant for virus replication but might come into contact with the RNA during the preparation of the cytoplasmic extract.

First, the efficiency of the UV cross-linking method for covalently binding viral RNA to protein was tested. In infected, dactinomycin-treated cells, the viral RNA was labeled with [$^3$H]uridine and half of the cells were UV irradiated. After dissociation by guanidinium thiocyanate, the two cytoplasmic extracts were separately centrifuged through cesium chloride. In the pellet, RNP was demonstrated and distinguished from free RNA with the protein-RNA filter binding assay (48). Nonspecific binding of free RNA, isolated from cells not cross-linked, was usually in the range of 0.2% of total radioactivity, whereas 9 to 10% of total radioactivity from UV-cross-linked cells was found to bind to the filter, indicating that this percentage of viral RNA was present as covalently bound RNP.

In order to identify viral proteins bound to RNA, pelleted RNP prepared with the guanidinium-cesium chloride method described above was digested with RNase and subjected to PAGE, and the proteins were reacted with MAb on Western blots. For Western blots with 2C-MAb, the equivalent of $3 \times 10^8$ cells and, for Western blots with anti-capsid MAb, $3 \times 10^7$ cells were optimal for one slot of a polyacrylamide gel. As a positive control, MAb against the capsid protein VP1 was used. This protein is in permanent close contact with the viral RNA within the virus particle (28, 36) and can thus be expected to become efficiently cross-linked to RNA. This is demonstrated in Fig. 1A, which shows that VP1 is present in the cesium chloride pellet obtained from UV-irradiated cells (lane 3). The protein shows a retardation in migration as compared with VP1 in a cytoplasmic extract from cells not UV cross-linked (lane 4). Such a retardation was described previously (6) as the result of nucleotides covalently bound to the protein and therefore being protected against digestion by RNase. Because of the irregular arrangement of the RNA within the virus particle, the VP1 molecules in one virion may have different numbers or lengths of contact sites with the RNA, and thus the VP1 moiety carries (after cross-linking) a heterogeneous amount of protected nucleotides. To show that the viral protein detected on the Western blot is bound to RNA, aliquots of a cesium chloride pellet obtained from UV-cross-linked cells were digested for various times with RNase before electrophoresis (20). With decreasing digestion, the amount of RNP entering the gel also decreases and the retardation of some of the VP1 molecules increases (Fig. 1B). The observations that the cesium chloride pellet from cells not UV irradiated contains no VP1 (Fig. 1A, lane 2), no VP0 and VP2 (not shown), no P2

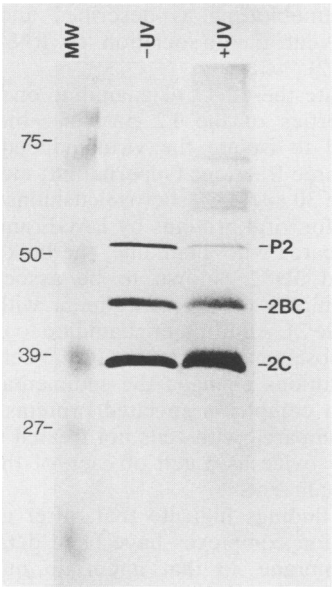



FIG. 3. Immunoblot of cytoplasmic extracts of UV-irradiated (+UV) cells or cells not UV irradiated (−UV). Immune reaction with 2C-MAb. A certain amount of the P2 proteins show retardation in migration and are thus considered cross-linked to RNA. The retardation was visible only when longer-than-normal (14 instead of 9 cm) gels were used. MW, Prestained molecular weight standard.

proteins (Fig. 2, lane 3), and no radioactivity when the proteins are previously radiolabeled (not shown) indicate that the RNA and RNP isolated by the guanidinium-cesium chloride method are not contaminated by free protein. This is also shown by spectrophotometric measurements of the isolated RNA which yielded optical density ratios of 260 and 280 nm of 2.06 to 2.2

Incubation of a Western blot with 2C-MAb revealed that the P2 proteins 2C, 2BC, and P2 can also be found in the RNA pellet after UV cross-linking (Fig. 2, lane 4). Again, retardation in migration is visible, and the proteins migrate in more than one band. A possible explanation is that there are several defined numbers of RNA attachment sites in a P2 polypeptide, leading to distinct amounts of protected nucleotides.

To estimate the amount of cross-linked viral P2 protein, Western blots were prepared from crude, RNase-digested cytoplasmic extracts of cross-linked cells and cells not cross-linked (Fig. 3). Incubation with 2C-MAb indicates that most of the individual P2 proteins from both cytoplasmic extracts migrated to the same distance, and only a fraction of 2BC and 2C in the cross-linked extract shows some retardation in migration. Together with the relatively large amount of cells needed to produce a signal in lanes 4 and 7 of Fig. 2, this means that only a fraction of these two proteins can be cross-linked to RNA, and the remainder is not (or is not permanently) RNA associated in the infected cell.

To demonstrate that the P2 proteins are bound to viral RNA, cells were treated with dactinomycin, and viral RNA was then selectively labeled with [$^3$H]uridine. RNA and RNP were isolated by the guanidinium-cesium chloride method described above, and the pellet not treated with nuclease was then immunoprecipitated with Sepharose-bound 2C- and VP1-MAb. The MAb precipitate radioactivity (Table 1), i.e., RNP composed of VP1 or P2 proteins and labeled (viral) RNA. If UV cross-linking was omitted, the background binding of labeled RNA was 5 to 10% of the bound radioactivity from cross-linked cells.

**Localization of the P2 proteins in the viral replication complex and on the membranes of virus-induced vesicles.** To learn more about the biological significance of the association of the P2 proteins with viral RNA, we studied the spatial arrangement of the P2 proteins within the replication complex and on the vesicular membranes. This was done by IEM, using isolated, virus-induced, and replication complex-carrying vesicles because such subcellular fractions are very suitable for high-resolution EM in negatively stained preparations. The virus-induced vesicles were isolated by centrifuging a cytoplasmic extract of poliovirus-infected cells onto a double cushion of 30 and 45% sucrose. The visible bands in both sucrose cushions were harvested separately, and the RNA-synthesizing capacity, the content of viral proteins, and the EM morphology of each fraction was assayed.

The vesicles from the 30 and 45% sucrose readily synthesized RNA in a dactinomycin-containing in vitro transcription system (40) (Fig. 4). This indicates that the method of vesicle isolation retains functionality of the replication complex and that, therefore, the spatial arrangement of the RNA replication machinery must not be severely altered. Preparation artifacts were also ruled out by examining 30 and 45% sucrose fractions embedded, sectioned, and processed for IEM as described elsewhere for whole cells (2). By this method, the arrangement of vesicles and replication complexes as well as the location of P2 proteins in the isolated vesicle fractions made no difference to the morphology and P2 protein location in the vesicle area of intact, infected cells (2) (not shown).

Negatively stained IEM preparations of the 30 and 45% fraction showed that both contain smooth-surfaced vesicles with the replication complex bound to their outer surfaces (Fig. 5A). Typically, the vesicles and the replication complexes form rosettelike structures with the replication complex in the center. The immunolabeling with 2C-MAb shows that the P2 proteins are contained within the replication complex but tend to accumulate at the border of this structure where it comes into close contact with the vesicu-

TABLE 1. Immunoprecipitation of RNP with MAb against protein 2C and VP1

| Treatment of cells | Immunomatrix with 2C-MAb | | Immunomatrix with VP1-MAb | |
|---|---|---|---|---|
| | Radioactivity (dpm[a]) added | Radioactivity (dpm) precipitated | Radioactivity (dpm) added | Radioactivity (dpm) precipitated |
| No UV irradiation | 417,195 | 178 (0.042%)[b] | 438,920 | 504 (0.11%) |
| UV irradiation | 406,530 | 3,413 (0.84%) | 387,712 | 3,645 (0.94%) |

[a] Viral RNA labeled with [$^3$H]uridine. At 3.75 h p.i., the cells were processed by the guanidinium-cesium chloride method to isolate RNA and RNP. dpm, Disintegrations per minute.

[b] In parentheses, percent precipitated of added radioactivity.

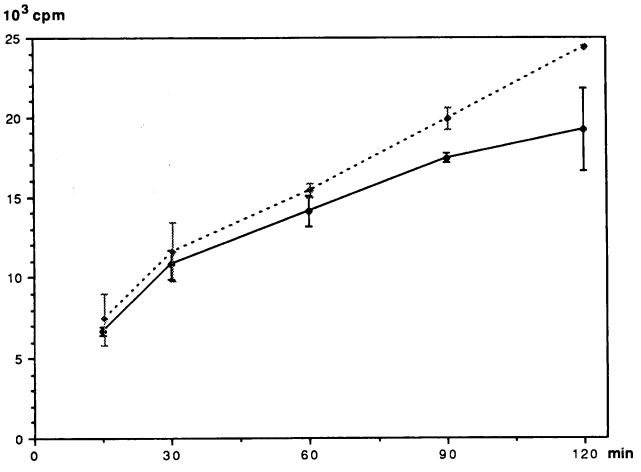



FIG. 4. Kinetics of the in vitro RNA synthesis of the 30% (——) and 45% (– – –) sucrose fractions. A cytoplasmic extract of infected HEp-2 cells was prepared at 4 h p.i. The vesicle-attached replication complexes were isolated by sucrose step gradient centrifugation, and fractions were assayed for dactinomycin-resistant RNA synthesis (38).

lar membrane. The membrane-binding property of the P2 proteins is further illustrated by the observation that they can also be found on the vesicular membrane at places where no replication complex is present. Figure 5B shows a rosette of replication complex and vesicles taken from an in vitro transcription system as described above. The IEM preparation was made after 60 min of ongoing in vitro RNA synthesis. The picture shows essentially the same features as Fig. 5A does.

The vesicles of the 45% sucrose fraction differ from those of the 30% fraction in that they have virus particles attached to their surfaces, which might explain the difference in sedimentation behavior. Details on the interaction of capsid proteins with the replication complex and the vesicular membranes during virus maturation will be published elsewhere (manuscript in preparation).

**Influence of guanidine hydrochloride on the association of P2 proteins with viral RNA, the replication complex, and the vesicular membrane.** Processes which can be blocked by guanidine are directly or indirectly dependent on the protein 2C (see above). To learn more about the function of the protein 2C, we tested whether guanidine would inhibit the association of the protein 2C with one of its counterparts (i.e., viral RNA, replication complex, or vesicular membranes).

To test the association of P2 proteins with viral RNA under guanidine, 2 mM guanidine hydrochloride was added to the infected cultures at 3 h p.i.; after UV cross-linking at 3.75 h, the RNPs were isolated and subjected to PAGE and Western immunoblotting as described above. Guanidine does not prevent the association of RNA with the P2 proteins (Fig. 2).

To investigate the effect of guanidine on the membrane-binding properties of the P2 proteins, the sucrose step gradient (used to isolate the virus-induced vesicles) was divided into three fractions (supernatant; membranes, combined from the 30 and 45% sucrose cushions; and sediment) and analyzed for viral proteins by PAGE and fluorography. In cells not treated with guanidine, the bulk of the proteins 2BC, 2C, and $3D^{pol}$, known to be associated with the replication complex (2, 4), cosediment with the vesicular fractions (Table 2). Addition of guanidine to the infected cell culture and subsequent processing of the cells in guanidine-containing solutions changed the sedimentation pattern of the replication complex-associated proteins 2BC, 2C, and 3D so that, compared with cells not treated with guanidine, approximately twice as much of each of these proteins is found in the sediment.

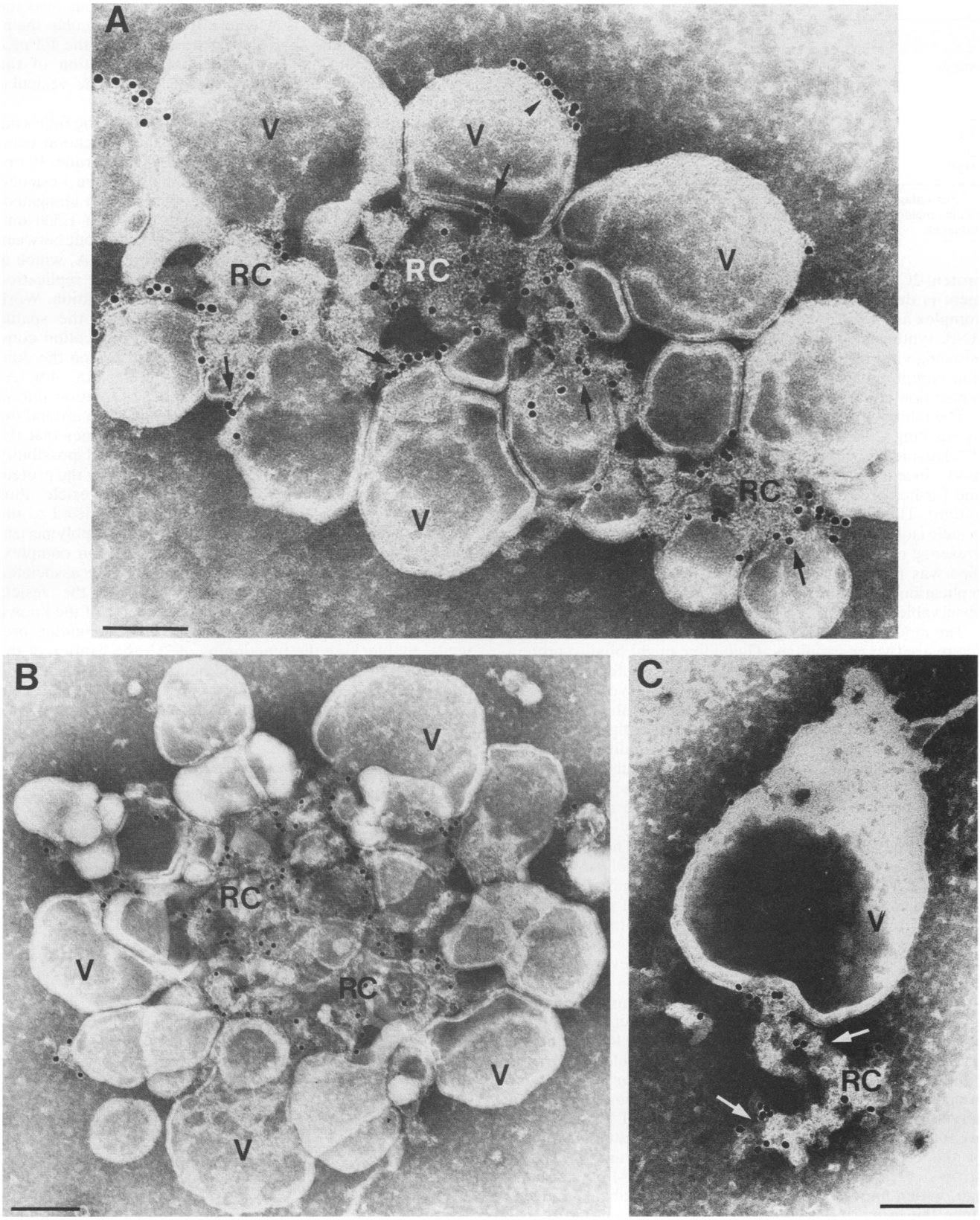
The above findings indicate that, after guanidine treatment, replication complexes have been detached from the vesicular membrane so that a certain number of them become sedimentable through the sucrose layers. This was also seen by EM and IEM of negatively stained specimens of the sucrose gradient fractions. Figure 5C shows a vesicle preparation from the 30% sucrose fraction of guanidine-treated cells, processed in the presence of guanidine. The rosettelike arrangement of vesicles around the replication complexes and thus the configuration of the replication complexes are no longer maintained in such preparations. Some replication complexes appear still loosely associated with only one or two vesicles (Fig. 5C), and most vesicles are free and dispersed. Labeling with MAb shows that the P2 proteins are still associated with the replication complex but no longer with the vesicular membranes.

## DISCUSSION

Poliovirus RNA synthesis depends on viral proteins such as the RNA polymerase $3D^{pol}$ (17, 25), the protein 2C (24), and the primer VPg-pU-pU (39, 40, 44) as well as on the specialized cellular membranes which render the components of the transcriptional machinery functional (16, 41). These membranes, originating from the rER (2), form numerous vesicles 200 to 400 nm in diameter. On their outer surfaces, they carry the viral replication complex with the viral RNA and the P2 proteins (2).

The membranes and replication complexes can be isolated in a transcriptionally active state by sucrose gradient centrifugation. Negatively stained IEM preparations showed that the fractions consist of rosettelike structures with peripheral vesicles and a central, elongated, stretched-out replication complex. The shape of the replication complex is maintained by the protein 2C (or its precursor[s]) attaching the replication complex to several vesicles. If the function of

FIG. 5. (A) Negatively stained, poliovirus-induced vesicles (V) (30% sucrose fraction) isolated by sucrose step gradient centrifugation at 4.5 h p.i. The immunocytochemical label is 2C-MAb 11/2B1.1 and goat anti-mouse antibody coupled to colloidal gold of 10 nm. The replication complex (RC) is firmly attached to the vesicular membranes. P2 proteins are associated with the vesicular membrane (arrowhead) or with the replication complex as well as with the membrane (arrows). Bar represents 100 nm. (B) Vesicles (V) and replication complex (RC) (30% sucrose fraction) from an in vitro transcription system, incubated for 60 min at 30°C. Preparation for immunoelectron microscopy was as described for panel A. The vesicles are in a rosettelike arrangement around the replication complex. P2 proteins are associated with both replication complex and vesicular membranes. Bar represents 100 nm. (C) Isolated virus-induced vesicle, 4.5 h p.i., as in panel A, but prepared from cells which were guanidine treated at 2.75 h p.i. and processed for IEM in guanidine-containing solutions. The rosette is disrupted, and the replication complex (RC) is only loosely associated with one vesicle (V). The P2 proteins are still associated with the replication complex but no longer bound to the vesicular membrane (arrows). Bar represents 100 nm.


A
V
V
RC
RC
V
V
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B
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RC
V
V
V
C
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RC

TABLE 2. Distribution of the viral proteins 2BC, 2C, and $3D^{pol}$ in the sucrose step gradient used for isolation of the vesicular fraction

| Protein | Amt of protein in each fraction[a] with: | | | | | |
|---|---|---|---|---|---|---|
| | No guanidine added | | | 2 mM guanidine added at 2.75 h p.i. | | |
| | Sup | Memb | Sed | Sup | Memb | Sed |
| 2BC | 0 | 76 | 24 | 0 | 44 | 56 |
| 2C | 3 | 70 | 27 | 2 | 51 | 47 |
| $3D^{pol}$ | 32 | 50 | 18 | 25 | 34 | 41 |

[a] Percentage of each protein in the entire gradient. Sup, Supernatant; memb, membrane fraction (30 and 45% sucrose combined; see text); sed, sediment.

protein 2C is blocked by guanidine, this rosettelike arrangement is destroyed, (as the P2 proteins and the replication complex are lost from most of the membranes), and the viral RNA synthesis comes to a halt. Thus, the functional entity allowing viral transcription to proceed consists of a replication complex and the surrounding vesicles which hold the replication complex in its proper configuration.

The inhibitory action of guanidine can be rapidly reversed by washing (29). Unfortunately, this prevents localization of [$^{14}$C]guanidine within the cell (e.g., by EM autoradiography), since the drug is completely washed out during fixation and further processing of the cells (our unpublished observation). This finding prompted us to perform all steps of the vesicle isolation and immunocytochemical preparation in the presence of guanidine, since otherwise the effect of guanidine was reversed and the loss of the P2 proteins and the replication complex from the membranes was no longer observable.

The mode of action of guanidine, however, is not known. There are two possibilities. Guanidine might bind to protein 2C and thus prevent (e.g., by changing conformational or electrochemical properties) its binding to the vesicular membrane. Alternatively, guanidine might bind to the membranes (or certain membrane components) and alter their properties in such a way that 2C cannot (or, with guanidine-dependent virus strains, is able to) bind to the vesicular surface. It cannot be decided at present which hypothesis is correct.

The P2 proteins are in a close association not only with membranes but also with viral RNA, so they can be cross-linked to the RNA by UV irradiation. This is not surprising, since the protein 2C was found to be involved in RNA synthesis (24). Although immunocytochemistry of sectioned, whole cells showed that virtually all of the P2 protein is bound to the vesicular membranes and the replication complexes (2), only a fraction of each P2 protein, including 2C, can be cross-linked to RNA. There are several explanations possible. It might be that the association of protein 2C to viral RNA is temporally limited (e.g., because P2 proteins and RNA are moving relative to each other) or that the association is partially indirect (i.e., the viral RNA is bound to one P2 protein molecule and this molecule, in turn, is bound directly [or via another protein] to several P2 proteins attached to the vesicular membrane). This would also be in agreement with the observation that several P2 molecules are found grouped together at one presumed attachment site of the replication complex on the membrane (Fig. 5A, arrows). (The amount of gold grains in the IEM preparations can, within limits, be used to quantitate the labeled antigen, since the primary antibody used is monoclonal and recognizes only one epitope.)

The exact nature of the interaction of the P2 proteins with the viral RNA is not known. From our observations that guanidine separates not only the P2 proteins but also the replication complex as a whole from the vesicular membrane, we conclude that, during transcription, the P2 proteins are responsible for the spatial organization of the replication complex and thus viral RNA on the vesicular membrane.

From the observations reported, we propose the following hypothesis to explain the structure of the replication complex and its interaction with the vesicular membrane. In our EM pictures of the isolated, functionally active vesicular fraction, the replication complex appears as an elongated, continuous structure of approximately 1,000 to 1,500 nm, which seems to be suspended on and stretched out between several vesicles. This would allow the viral RNA, which is 2,700 to 2,800 nm in length (27), to fit into the replication complex in a more or less stretched out configuration. Work is in progress in our laboratory to visualize the spatial configuration of the viral RNA within the replication complex. Viral plus-strand RNA synthesis initiates on the double-stranded, helical, replicative-form RNA (18, 40). As pointed out previously (21), this implies a rotation during transcription, either of the growing progeny RNA around the template or of the replicative form, if one assumes that the progeny strands are fixed. Clearly, the second possibility seems more feasible (21). It could be achieved by the protein 2C attaching each progeny RNA strand to a vesicle, thus preventing the progeny RNA to become hybridized to the template, which is turning itself (driven by the polymerization reaction) lengthwise through the replication complex. The progeny RNA would then be immediately associated with viral capsid material on the surface of the vesicle (manuscript in preparation). Conversely, some of the known effects of guanidine can thus be explained: guanidine prevents, by blocking the functioning of 2C, the fixation of the progeny RNA to the vesicle and hence prevents its dissociation from the template, which, consequently, inhibits encapsidation (47), stops initiation of a next RNA strand (30), and leads to an accumulation of the replicative form (9).

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