Intermediates of Bacteriophage MS2 Assembly In Vivo

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The in vivo process of virion assembly was studied in rifampin-treated, MS2-infected *Escherichia coli* during late times of infection—after 18 min postinfection. Differential sucrose gradient sedimentation of infected-cell lysates taken at various times after radioactive labeling indicated a definite temporal order of appearance of phage-specific protein in assembly-related structures. Labeled MS2 protein appears first as a low-molecular-weight peak at the tops of gradients, then as a peak at 40S and as a large number of almost unseparable structures between 40 and 80S, and finally as 80S mature phage particles. During the chase of a short labeling period, radioactive phage protein was found to disappear from gradients in the same temporal order as it appeared; the soluble peak disappears first, followed by the 40 to 70S region. The chased label appears quantitatively in the 80S phage peak. Labeled phage RNA was found to appear first in the 40S peak, then in the structures between 40 and 70S, and finally in 80S phage particles. The order of disappearance of labeled phage RNA during a chase is the same as its appearance. Resedimentation of the 40 to 70S region indicated the presence of distinct structures at 60 and 70S and many indistinct ones between 40 and 60S. The smaller intermediates exhibit separable maturation protein-rich and coat protein-rich segments, indicating nonrandom binding of the two proteins during the initial steps of assembly. Larger, discrete intermediates appear at 60 and 70S. Treatment of the various structures with pancreatic RNase results in destruction of those from 40 through 60S; treatment of the 70S structure results in the conversion of some of it to a 45S peak, presumably the complete capsid. A small fraction of the 80S phage peak is also sensitive to RNase, resulting in a similar 45S peak. Pulse-chase experiments indicate that structures from 40 through 60S as well as the RNase-sensitive 70S structure are assembly intermediates, but that the RNase-insensitive 70S and the RNase-sensitive 80S structures are not.

MS2 is one of a group of small icosahedrally symmetrical, RNA-containing viruses which infects male *Escherichia coli*. The virion consists of a single linear molecule of RNA, 180 coat protein subunits, and a single molecule of maturation protein (21, 22). A phage-specific RNA polymerase is found in infected cell lysates (12).

Much work has been done on the possible mechanisms involved in in vitro assembly of RNA bacteriophages (7, 20). Virus-like particles which are of the same size as, and have many physical properties in common with, authentic virus particles, can be obtained from reaction mixtures containing only phage RNA and coat protein (6, 23). These reconstituted particles lack the maturation protein, are sensitive to RNase digestion, are not infectious, and are

similar in all respects to the maturation protein-deficient defective particles identified by Steitz (21). Under proper conditions, however, infectious virus particles can be produced in vitro from reaction mixtures containing maturation protein in addition to the coat protein and RNA (8, 15). These in vitro-produced, infectious R17 particles sedimented at 80S with a shoulder at 70S and were sensitive to RNase digestion (15), indicating an imperfectly assembled particle.

In addition to mature virus particles, various other phage-related structures are, or can be, produced in vivo during infection of susceptible host bacteria. These include the 70S defective particles produced during infection of a nonpermissive host by phage carrying an amber mutation in the maturation protein cistron (21) as well as defective particles produced by depression of maturation protein synthesis relative to coat protein synthesis by removal of histidine

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from the infected cell culture medium (9). Addition of histidine to histidine-starved, infected cultures did not result in production of infectious phage from defective particles. Thus, to obtain infective particles, maturation protein must not only be present in the completed capsid but must also be in early and intimate association with the assembly process.

Examination of MS2-infected cell lysates at late times of infection has revealed the presence of 40 to 45S structures which contain phage RNA and all three phage proteins (1, 4, 5, 14). It has been suggested that some or all of these structures represent the first intermediate of *in vivo* phage assembly. The assembly process has been visualized as beginning on RNA molecules which are part of a replication-translation complex consisting of replicative intermediates with ribosome-bound, translation-regulated, single-strand RNA tails (1, 7, 14). The start of assembly may be signalled by the binding of coat protein to these RNA-protein complexes, in excess of that required for regulation of translation, along with the binding of one or more molecules of maturation protein; the result being an assembly intermediate of 40 to 45S. The maturation protein could bind near the 5' end of the RNA molecule (9) and may play a role in inducing and maintaining a secondary structure of the RNA necessary for continued assembly and capsid completion (1). Assembly can continue through addition of postulated coat protein capsomeres to this first intermediate. The nature of the coat protein capsomeres is not clear; they could be monomers, dimers (16, 17), trimers, or even pentamers and hexamers (26).

This paper is concerned with studies on the *in vivo* assembly process of MS2. During "late" times of infection (after 18 min postinfection [p.i.]) there is a flow of labeled phage proteins from low-molecular-weight subunits to a 40S peak similar to that discussed above, and to various structures which sediment between 40 and 70S, and, finally, to 80S mature phage particles. The 40S peak and the 40 to 70S structures are believed to represent intermediates in the assembly process, and experiments designed to reveal some of the properties of these intermediates are described.

MATERIALS AND METHODS

[³H]leucine (58 Ci/mmol), [³H]histidine (30 Ci/mmol), [³H]uracil (19.4 Ci/mmol), and a ¹⁴C-labeled amino acid mixture (containing ala, arg, asp, glu, ilu, leu, lys, phe, pro, ser, thr, tyr, and val) were purchased from Schwarz Bioresearch; [¹⁴C]uracil (0.5 mCi/mg) was purchased from New England Nuclear

Corp.; and rifampin was purchased from Mann Research Laboratories.

TES is 0.03 M Tris-hydrochloride, pH 7.2, 0.005 M EDTA, and 0.10 M sodium chloride. SSC (1×) is 0.15 M NaCl plus 0.015 M sodium citrate adjusted to pH 7.2. MMT medium consists of solution 1 (13 g of Trizma base, 2 g of ammonium ohloride, 6.7 g of monosodium glutamate, and 350 mg of K₂HPO₄ dissolved in 200 ml of distilled water), solution 2 (0.4 g of MgSO₄·7H₂O, 10 mg of vitamin B₁, and 85.2 mg of calcium chloride dissolved in 200 ml of distilled water), and solution 3 (10 g of dextrose dissolved in 200 ml of distilled water). MMT medium is prepared by adjusting the pH of solution 1 to 7.2, separately autoclaving the three solutions, and mixing when cool.

To prepare stocks of phage, 150 ml of *E. coli* C3000 at 2×10^8 to 5×10^8 cells per ml in MMT medium plus 0.2% Casamino Acids were infected with MS2 at an MOI of 5 to 10. The infected cells were allowed to incubate at 37 C with shaking for 2 h. Chloroform (1 ml/liter) was added and shaking was continued for 30 min at 37 C. The lysate was cleared by centrifugation at $10,000 \times g$ in a Servall SS-34 rotor and the supernatant was centrifuged at $80,000 \times g$ in a Spinco no. 30 rotor for 4 h to pellet the phage particles. The pellet was homogenized in 6 ml of 1× SSC and dialyzed against two changes of SSC. The dialysate was cleared by centrifugation. Titers of 10^{11} PFU per ml were obtained.

Sucrose gradients were either 6 to 20% or 15 to 30% sucrose in TES. A Spinco SW50.1 rotor was used at 50,000 rpm for 80 min (6 to 20% gradients) or 120 min (15 to 30% gradients) at 3 C in a Spinco model L2 ultracentrifuge in all experiments reported. When a portion of a gradient was to be recentrifuged it was immediately diluted 1:1 with TES to ensure layering on the secondary 15 to 30% gradient. When gradients were assayed directly for radioactivity they were dripped onto 1½ inch squares of Whatman 3 MM filter paper. If only a portion of a gradient was to be assayed then a small portion of each fraction was pipetted onto filter paper squares. The squares were washed once in ice-cold 5% trichloroacetic acid and twice in ice-cold 80% ethanol. They were dried in an oven and counted in a Liquifluor-toluene scintillation counting cocktail.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The method of Maizel (11) as modified by Kiehn and Holland (10) was used. Solution 1 is 30% (wt/vol) acrylamide (electrophoresis grade, Eastman Kodak) plus 1% (vol/vol) ethylene diacrylate (EDA, Monomer-polymer Lab, Borden Chemical Co.). Solution 2 is 0.3% (vol/vol) tetraethylmethylenediamide (TEMED, Eastman Kodak). Solution 3 is 0.4 M Tris-acetic acid buffer, pH 9.1, 2.0 M urea, 0.4% (wt/vol) SDS, 0.004% (wt/vol) EDTA, and 0.4% (vol/vol) betamercaptoethanol (BME). Solution 4 is 0.3% ammonium persulfate, freshly prepared for each use. Acrylamide gels (7.5%) were prepared by mixing equal volumes of solutions 1, 2, 3, and 4 by gentle rotation. The gel solution was then added to capped, acid washed 6-mm diameter glass tubes to a depth of 18 cm. Tray buffer (1×) (0.1

M Tris-acetic acid buffer, pH 9.1, 0.05 M sodium acetate, 0.1% SDS, 0.001% EDTA, and 0.09% thioglycolate) was then added as an overlay to provide a flat surface and to ensure gelation. Sixty minutes were allowed for the gels to harden. The top of the gel was then rinsed with 1× tray buffer and the tube placed in the electrophoresis apparatus. To electrophorese thioglycolate into the gel so that a reducing atmosphere would exist when the sample was applied 185 V were applied to the gel for 10 to 15 min. The top of the gel tube was then drained and the sample in 1× sample buffer plus bromphenol blue marker dye was applied. Sample buffer (1×) is 0.01 M Tris-acetic acid buffer, pH 9.1, 0.1% SDS, 0.001% EDTA, 0.5 M urea, and 0.1% BME. Tray buffer (1×) was then layered above the sample and the top tray was refilled. Twenty to 30 V were then applied for 30 min to focus the sample and 185 V (electrophoresis voltage) were applied until the dye marker neared the end of the tube (about 4.5 h). After electrophoresis the gels were removed from the tubes and manually sliced into 2-mm fractions with a razor blade. The slices were then added to 10 ml of Liquifluor-toluene scintillation fluid containing 3.5% NCS solubilizer (Amersham-Searle) in a 15-ml scintillation vial. The vials were capped tightly and swirled vigorously on a large rotary shaker at 37 C until the slices were dissolved (8 to 10 h). They were then counted in Beckman LS series scintillation counters. Bacteriophage ϕ X-174 proteins were used as molecular weight markers for electrophoresis. They were kindly donated by D. H. Gelfand and consisted of A protein (mol wt 40,000 to 44,000), E protein (mol wt 48,000 to 52,000), and F protein (mol wt 19,000 to 21,000) (personal communication). [14 C]dimethylsulphate-labeled bovine serum albumin (67,000 mol wt) and ovalbumin (45,000 mol wt) were also used as markers and were the gift of M. Doyle.

Preparation of samples for SDS-gel analysis. Samples were routinely taken from sucrose gradients and prepared for gel electrophoresis. If the sample was greater than 0.5 ml, it was first concentrated with Lyphogel (Gelman) to 0.25 to 0.30 ml. Sample buffer (10×) and 100% BME were then added to the sample to give final concentrations of 2× and 0.5%, respectively. The sample was then heated to 100 C for 5 min, cooled, and dialyzed overnight against 1× sample buffer and 0.1% BME in 8 M urea. Bromphenol blue was added after dialysis and just before electrophoresis.

In a typical experiment *E. coli* C3000 was grown to 2×10^8 to 5×10^8 cells per ml in MMT at 37 C with shaking. Samples (5 ml each) of cells were transferred to test tubes at 37 C and aerated vigorously. After 5 min of aeration MS2 were added at an MOI of 5 to 10. At 5 min p.i. rifampin was added to 100 μ g/ml. Aeration was continued and at 2 min before labeled leucine, histidine, or uracil was to be added, the corresponding unlabeled compound was added to low concentration to ensure linear label incorporation for the time period desired. Amounts of unlabeled material added ranged from 0.02 μ g/ml to 0.5 μ g/ml for linearity from 2 to 20 min. When the 14 C-labeled amino acid mixture was to be used, no unlabeled coun-

terpart was added; linear incorporation could be achieved with the labeled mixture alone. At the appropriate time, label was added, and in chase experiments, the unlabeled counterpart was added at the indicated time. Cells were allowed to continue growth with aeration, and 5-ml samples were taken at various times, chilled quickly to 0 C in a dry ice-methanol bath, and kept on ice until all samples were collected. They were then centrifuged at $6,500 \times g$ in the Sorvall SS-34 rotor at 0 C and washed once with ice-cold MMT medium plus the unlabeled counterpart to the isotope used. The washed cells were lysed by the method of Godson and Sinsheimer (4). They were resuspended in 0.10 ml of 20% sucrose in 0.1 M Tris-hydrochloride, pH 8.1, 0.02 ml of 27 mg/ml EDTA and 0.02 ml of 100 μ g/ml lysozyme and allowed to form protoplasts for 5 min at 0 C. They were lysed by pipetting into 0.1 ml 5% BRIJ-58 (Atlas), 0.03 ml 1% deoxycholate, 0.03 ml 0.1 M $MgCl_2$, and 0.01 ml of 1 mg/ml electrophoretically pure DNase (Worthington). The lysate was then layered immediately on the sucrose gradient and centrifuged.

Sucrose gradient fractions which were to be treated with pancreatic RNase (Worthington) before resedimentation were diluted 1:1 with RNase dissolved in TES and incubated at room temperature for 15 min. The final RNase concentration was 20 μ g/ml. A final concentration of 50 μ g/ml gave results identical to 20 μ g/ml. After incubation, the samples were chilled, layered onto 15 to 30% sucrose gradients in TES, and recentrifuged.

RESULTS

Rifampin inhibits bacterial RNA and protein synthesis almost completely (13) by combining with the DNA-dependent RNA polymerase (25) and preventing initiation but not propagation of RNA synthesis (19). If rifampin is added at least 5 min after infection of *E. coli* by bacteriophage f2 the drug has no effect on phage production (2). When MS2-infected, rifampin-treated cultures of *E. coli* C3000 are radioactively labeled at late times of infection (18 min p.i. or later), phage-specific protein is found, after lysis and sucrose gradient sedimentation, in the small-molecular-weight (soluble) region, a peak at 40S, between the 40S peak and 80S, and as 80S mature phage (Fig. 1); phage RNA appears only in the three heavier regions. Rifampin-treated, uninfected cultures display a minor peak of labeled protein only in the soluble region of sucrose gradients and no labeled RNA at all. Similar results have been obtained in actinomycin-D treated cells (14).

Purified MS2 RNA was added to uninfected and to 26-min infected, rifampin-treated cell lysates to determine whether the 40S complex could be formed after lysis. The RNA was added to the lytic mixture so that it would be present as the cells were lysing. It was found that the RNA suffered no degradation and sedimented

FIG. 1. Infected cells labeled with 1 μ Ci of 14 C-labeled amino acid mixture per ml at 25 min p.i. Samples taken for 6 to 20% sucrose gradient sedimentation at: ($\blacktriangle$), 30 s; (O), 1 min; ($\triangle$), 2 min; and ($\bullet$), 5 min after addition of label.

as free 29S RNA with a shoulder corresponding to about 35S in both lysates. Godson and Sinsheimer (4) considered a similar 35S peak to be MS2 RNA attached to a 30S ribosome subunit. No 40S material could be detected.

To determine whether precursor-product relationships exist among the four phage-specific regions, continuous label and pulse-chase experiments were undertaken. Cells labeled at 20 min p.i. for varying periods of time with 1 μ Ci/ml of a 14 C-labeled amino acid mixture showed, after centrifugation, that the soluble region was labeled first (within 30 s of label addition), followed closely by the 40S peak and the 40 to 70S region (within 2 min) and finally the 80S mature phage peak (within 5 min) (Fig. 1). Similarly treated cells labeled with [3 H]uracil indicated that phage RNA appeared first at 40S and then in progressively heavier forms. Other experiments have shown that RNA begins to appear in 80S phage at 6 to 7 min after label addition and that there is never a distinct peak or shoulder of 29S viral RNA, as shown also by Cramer and Sinsheimer (1). When infected cells were pulsed with 0.2 μ Ci of 14 C-labeled amino acids per ml at 20 min p.i. and chased with 2% Casamino Acids at 22 min p.i. it was found that in samples taken after 1, 3, 6, 9, and 15 min of chase, labeled protein found in all regions of the gradient could be chased into 80S phage (Fig. 2). It can be seen, also, that the soluble re-

gion began to lose label within 1 min of chase, whereas the 40S peak and the 40 to 70S region began to lose label between 3 and 6 min of chase. Label is chased into the 80S phage peak within 1 min. Figures 1 and 2 together show that the order of appearance of protein label in the four gradient regions is the same as the order of disappearance during a chase period. The behavior of MS2 RNA during a pulse and chase experiment is similar to the protein behavior. Infected cells labeled at 20 min p.i. with [3 H]uracil and chased with unlabeled uracil from 21 min to 36 min p.i. indicate, after lysis and sucrose gradient centrifugation, that phage-specific RNA is chased from the 40S peak and the 40 to 70S region into 80S phage particles. To show that the flow of counts out of the 40S peak and into 80S phage seen in the above pulse-chase experiment reflects movement of actual MS2 proteins, the 40 and 80S peaks resulting from another pulse-chase experiment were analyzed by SDS-polyacrylamide gel electrophoresis. Infected cells were labeled at 18 min p.i. with 4 μ Ci of [3 H]leucine per ml and chased at 21 min p.i. with 200-fold excess unlabeled leucine. Samples (5 ml each) were taken at 21, 24, and 28 min p.i. (0-, 3-, and 7-min chase). These samples were then sedimented through 6 to 20% sucrose gradients and the 80 and 40S peaks were isolated and prepared for electrophoresis as described in Materials and Methods. As expected, the label in both coat protein and maturation protein from the 80S phage peak increased during the chase (Fig. 3A). The label in these two proteins from the 40S peak decreased during the chase but that in the polymerase remained unchanged (Fig. 3B). Polymerase is not chased presumably because its synthesis is stopped at about 20 min p.i. (12, 24). That the coat protein and maturation protein peaks from the 7-min chase sample of Fig. 3B do not coincide with the peaks from the other samples is due to inadvertent stretching of the unstable EDA-cross-linked gels during slicing.

When samples are taken from a sucrose gradient of an infected-cell lysate and resedimented through sucrose gradients, the resulting peaks appear at approximately their original S-value and indicate the presence of a series of structures which might be intermediates of phage assembly. In such a resedimentation experiment, infected cells were labeled with 20 μ Ci/ml each of [3 H]histidine from 14 to 21 min p.i., lysed, and sedimented through a 6 to 20% sucrose gradient. Samples were taken from this gradient at 80 to 83S, 65 to 67S, 57 to 60S, 50 to 53S, 43 to 45S, diluted with TES, and resedi-

FIG. 2. Infected cells were labeled with $0.2 \mu\text{Ci}$ of ^{14}C -labeled amino acid mixture per ml at 20 min p.i. The pulse label was chased by addition of Casamino Acids to 2% at 22 min p.i. The ordinate is the percentage of counts appearing in each fraction relative to the total number of counts recovered on each gradient. The inset shows the effectiveness of the chase as counts per 0.1 ml of infected culture which are precipitable by cold 5% trichloroacetic acid after incubation in 0.5 N KOH at 37 C for 5 h. The arrows at the top of the figure represent the position of, from left to right, marker MS2 phage (80S) and MS2 RNA (29S). Samples (5 ml) of the infected culture were taken for sucrose gradient analysis at: (▲), 1 min; (△), 3 min; (○), 6 min; (□), 9 min; and (●), 15 min of chase.

mented. The five samples resedimented at approximately 80, 70, 60, 45, and 40S, respectively. The 50 to 53S original sample resedimented as two broad peaks, one at 45S and the other at 20S. The 43 to 45S original sample resedimented mostly as a broad 20S peak with a shoulder at 40S (Fig. 4).

When these various peaks were analyzed by SDS-gel electrophoresis, it was found that all

contained coat protein and maturation protein and, surprisingly, all but the 70 and 80S samples contained RNA polymerase (Fig. 5A, B, and C).

The two peaks resulting from the original 50 to 53S sample and the 20S peak from the original 43 to 45S sample showed very different compositions. The heavier peak (45S) from the first sample contained mostly coat protein and

FIG. 3. Infected cells were labeled at 18 min p.i. with 4 μ Ci of [3 H]leucine per ml and chased at 21 min p.i. with unlabeled leucine. The resulting lysates were sedimented through 6 to 20% sucrose gradients and the 40S peaks and the 80S phage peaks were isolated and prepared for SDS-gel electrophoresis as in *Materials and Methods*. (A) SDS-gel electropherograms of the 80S phage peak after: (O), a 3-min pulse; (●), 3-min chase; and (■), 7-min chase. (B) SDS-gel electropherograms of the 40S peak after: (●), a 3-min pulse; (O), 3-min chase; and (□), 7-min chase. The three peaks correspond to the molecular weights of, from left to right, phage RNA polymerase, maturation protein, and coat protein as determined by molecular weight markers described in *Materials and Methods*.

the lighter peaks (20S) from both samples contained mostly maturation protein and polymerase (Fig. 5C). The ratios of counts in coat protein, maturation protein, and polymerase are 35:4:1 for the 45S peak and 0.4:1:1 and 0.6:1:1 for the 20S peaks from the original 50 to 53S and 43 to 45S samples, respectively.

In another, similar experiment five samples (from 72 to 37S) were taken from a primary gradient of a protein and RNA-labeled lysate and resedimented. The samples resedimented at approximately their original *S*-value and all contained RNA.

The positions of these various intermediates on sucrose gradients should be interpreted in an operational sense only; that is, the *S*-value with which any structure sediments under these conditions can be influenced by factors other than the size of the phage-specific structure. For instance, since the samples applied to the gradients are cleared cell lysates, then even weak binding of the RNA portion of these intermediates to 30 and 50S ribosomal subunits could influence their final position on a gradient. Also, the RNA polymerase found in all intermediates of up to 60S can quite possibly be

part of RNA replication complexes and not be involved in assembly at all; these complexes, if present, could also affect the *S*-value of assembly intermediates.

To determine the extent to which the structure of these resedimented peaks depends upon RNA, samples taken from a primary gradient were treated with RNase before resedimentation (Fig. 6A to E). Infected cells were labeled from 20 to 25 min p.i. with 20 μ Ci of [3 H]leucine per ml, lysed, and sedimented through a 6 to 20% sucrose gradient. Single fraction samples were taken from this gradient at 83, 70, 60, 53, and 47S and were treated with 20 μ g of pancreatic RNase per ml for 15 min at room temperature before resedimentation. The 80S sample resedimented as two peaks; one at 80S (phage particles) and the other at 45S. The RNase-treated 70S sample also resedimented as two peaks; one at 70S and the other, again, at 45S. The RNase-treated 60, 53, and 47S samples behaved similarly upon resedimentation and displayed no distinct peaks except at very low *S*-values; their structure was essentially destroyed by RNase. It is likely the 45S peak resulting from RNase treatment of the 80 and

FIG. 4. Infected cells were labeled from 14 to 21 min p.i. with 20 μ Ci of [3 H]leucine per ml and 20 μ Ci of [3 H]histidine per ml. They were lysed and sedimented through a 6 to 20% sucrose gradient. Samples were taken at 80 to 83S, 65 to 67S, 57 to 60S, 50 to 53S, and 43 to 45S, diluted with TES, and immediately recentrifuged through 15 to 30% sucrose gradients. Symbols: $\circ$, the resedimented 80 to 83S sample; $\bullet$, 65 to 67S sample; $\times$, 57 to 60S sample; Δ , 50 to 53S sample; $\blacktriangle$, 43 to 45S sample.

70S samples represents the complete phage capsid; the capsid has been shown to sediment at about 45S and to be RNase resistant (6, 17, 22). The 45S structure described here is RNase insensitive, is not infectious, contains no RNA (Fig. 10), and is constructed of coat protein and maturation protein only (Fig. 11)—all characteristics expected of the capsid. These RNase experiments indicate that the 80 and 70S structures exist in RNase-sensitive and insensitive forms, and that both contain the complete phage capsid. The structures at 60S and smaller do not contain the complete capsid since they are destroyed by RNase and do not contain a full complement of coat protein (see discussion).

FIG. 5. (A) SDS-gel electropherogram of the 80S peak resulting from the 80 to 83S sample of Fig. 4. (B) SDS-gel electropherograms of the peak fractions from the resedimented 65 to 67S and 57 to 60S samples. Symbols: $\circ$, the 70S peak from the 65 to 67S sample; $\blacktriangle$, the 60S peak from the 57 to 60S sample. (C) SDS-gel electropherograms of the peak fractions from the resedimented 50 to 53S and 43 to 45S samples.

Symbols: Δ , the 45S peak from the 50 to 53S sample; $\bullet$, the 20S peak from the 50 to 53S sample; $\circ$, the 20S peak from the 43 to 45S sample. The arrows represent the expected position of, from left to right, MS2 RNA polymerase, maturation protein, and coat protein, determined by molecular weight markers as described in Materials and Methods.

Precursor-product relationships among the three largest structures (60, 70, and 80S) appearing on sucrose gradients of infected-cell lysates were examined by pulse and chase experiments at "late" times of infection. A 3-min [^3H]leucine pulse of infected cells at 18 min p.i. was followed by unlabeled leucine chase periods of 3 and 8 min. The three lysates were then sedimented through 6 to 20% sucrose gradients and the resulting 80, 70, and 60S pooled regions were diluted with TES and resedimented through 15 to 30% gradients (Fig. 7A to C). The behavior of the 60 and 80S samples was as expected for a precursor-product relationship—label at 60S is chased out while that at 80S is greatly increased. The 70S sample, however, does not act as an assembly intermediate because label present at the end of the pulse is actually increased somewhat by the chase.

Since a portion of the 70S form had been found to be sensitive to RNase degradation (Fig. 6) the previous pulse and chase experiment was repeated with the addition that the 70S samples were treated with 20 μg of pancreatic RNase per

ml for 15 min at room temperature before the second centrifugation. The results shown in Fig. 8 show that the fraction of RNase sensitive material (as measured by formation of capsid) decreases during the chase and that the RNase-insensitive fraction increases. The total amount of labeled material increases, again, only slightly during the 8-min chase. The 70S form was also examined after various periods of continuous labeling with [^3H]leucine. When the label was added at 18 min p.i. tritium was found to increase rapidly at 70S for 6 min and slowly to 11 min after labeling. The 70S form is noninfectious and contains RNA.

Since a portion of the 80S mature phage also was found to be sensitive to RNase, a separate experiment was carried out to investigate the behavior of the RNase-sensitive and insensitive forms during a chase of a 3-min pulse. Infected cells were labeled with 2 μCi of [^3H]leucine per ml at 18 min p.i. and chased at 21 min p.i. with unlabeled leucine. Samples were taken for sucrose gradient analysis at 0 min (3-min pulse), 3 min, and 8 min of chase. The 80S phage peaks were diluted 1:1 with TES and treated with 20

FIG. 6. Infected cells were labeled with 1 μCi of [^3H]leucine per ml from 20 to 25 min p.i., lysed and sedimented through a 6 to 20% sucrose gradient. Five samples were taken at 83, 70, 60, 53, and 47S. These samples were diluted with TES and treated with 50 μg of pancreatic RNase per ml for 15 min at room temperature. They were then resedimented and the gradient fractions analyzed for tritium counts. (A) The resedimented 83S sample; (B) the resedimented 70S sample; (C) the resedimented 60S sample; (D) the resedimented 53S sample; and (E) the resedimented 47S sample.

FIG. 7. Infected cells were labeled from 18 to 21 min p.i. with $2 \mu\text{Ci}$ of $[^3\text{H}]$ leucine per ml and chased from 21 to 24 and 29 min p.i. with unlabeled leucine. The resulting lysates were centrifuged through 6 to 20% sucrose gradients and the 80, 70, and 60S regions were individually pooled and resedimented through 15 to 30% sucrose gradients. (A) The sucrose gradient profiles of the resedimented 80S phage peaks; (B) the resedimented 70S sample; (C) the resedimented 60S sample. Symbols: Δ , 3-min pulse; $\bullet$, 3-min chase; $\circ$, 8-min chase.

μg of pancreatic RNase per ml at room temperature for 15 min before recentrifugation. Figure 9 indicates that after recentrifugation the fraction of 80S material which is insensitive to RNase increased greatly, whereas the sensitive form increased only slightly, if at all, during the chase. The fact that the radioactivity in the sensitive form remained practically constant during the chase could be a result of either the chase or cessation of its synthesis between 24 and 29 min p.i. To determine whether its synthesis is actually ended during this time period, infected cells were labeled at 15 min p.i. with $[^{14}\text{C}]$ uracil and at 18 min p.i. with $[^3\text{H}]$ leucine. Samples were taken at 21, 24, and 29 min p.i., lysed, and sedimented through 6 to 20% gradients. The 80S regions were isolated, treated with $20 \mu\text{g}$ of RNase per ml, and recentrifuged. Figures 10A and B are the recentrifugation profiles and indicate that the synthesis of the RNase-sensitive 80S particle is nearly ended by about 24 min p.i.; the increase from 24 to 29 min p.i. is quite small. It is also indicated that the 45S capsid does not contain RNA.

It was thought that the 70S structure found here could be the same as the maturation protein-deficient "defective particle" of Steitz. These particles are produced during infection of a nonpermissive host by a phage carrying an amber mutation in the maturation protein cistron. They sediment at 70S, contain no maturation protein, and are sensitive to RNase (22). If the 70S form found here during wild-type MS2 infection (especially the RNase-sensitive type) is the same as the "defective particle" then a pulse-chase experiment followed by SDS-gel analysis of maturation protein in the untreated 70S structure and the capsid produced by RNase treatment should confirm or deny the presence of "defective particles." Since capsid derived from virions contains maturation protein (18) then capsid derived from 70S would not be expected to lose it preferentially; that is, if no maturation protein can be found in these capsids it can be assumed there was also none in its 70S parent structure.

With this in mind infected cells were labeled from 18 to 23 min p.i. with $10 \mu\text{Ci}$ of $[^3\text{H}]$ leucine per ml. They were then lysed and centrifuged. The 70S region of the first sucrose gradient was isolated and divided into two parts. One was treated with RNase and both were resedimented. The resedimented 70S peak from the untreated sample and the capsid peak from the RNase-treated sample were individually pooled and electrophoresed on SDS-gels. Figure 11 indicates that both structures contain maturation protein and coat protein in essentially

FIG. 8. Infected cells were labeled from 18 to 21 min p.i. with $5 \mu\text{Ci}$ of $[^3\text{H}]$ leucine per ml and chased from 21 to 25 and 29 min p.i. with unlabeled leucine. The resulting lysates were centrifuged through 6 to 20% sucrose gradients and samples taken at 70S. These samples were treated with $20 \mu\text{g}$ of pancreatic RNase per ml and resedimented. Symbols: Δ , after a 3-min pulse; $\bullet$, after a 4-min chase; $\circ$, after an 8-min chase.

identical ratios. From these results it can be concluded that neither the RNase-sensitive nor the insensitive 70S structure lacked the maturation protein and that both are, in this respect, different than the "defective particle."

An attempt was made to quantitate the relative amount of maturation protein in the various structures sedimenting from 40 to 80S by comparing the radioactivity in maturation protein to the radioactivity in MS2 RNA at points along the gradient under conditions of constant specific activity. It was found that if $[^{14}\text{C}]$ uracil and $[^3\text{H}]$ leucine were added to infected cultures at 15 min p.i. then a constant level of labeled proteins and RNA could be achieved between 50 and 70S at 21 min p.i., a time when the level of 80S phage is increasing rapidly. It is assumed that the constant levels of

radioactivity in RNA and protein indicate that the intermediate structures between 50 and 70S are at their maximum concentration and that specific activity of the two labels is constant. Since MS2 RNA enters the assembly scheme at only the 40S peak, and if the above labeling conditions are attained, then the radioactivity appearing in MS2 RNA at any point between 40 and 70S should be a measure of the amount of any intermediate containing a single RNA molecule sedimenting at that point. Therefore, if there is a constant number of maturation protein molecules associated with each RNA molecule then the ratio of tritium in maturation protein to carbon-14 in RNA should be constant among samples taken from 50 to 70S.

Accordingly, cells were labeled from 14 to 21 min p.i. with $[^3\text{H}]$ leucine and $[^{14}\text{C}]$ uracil, lysed,

FIG. 9. Infected cells were pulsed with $2 \mu\text{Ci}$ of $[^3\text{H}]$ leucine per ml at 18 min p.i. and chased with unlabeled leucine at 21 min for 3 and 8 min. They were then lysed and sedimented through 6 to 20% sucrose gradients. The three 80S peaks were isolated, diluted with TES, and treated with $20 \mu\text{g}$ of RNase per ml. The three 80S samples were then resedimented through sucrose gradients. Symbols: Δ , 3-min pulse; $\bullet$, 3-min chase; $\circ$, 8-min chase.

and centrifuged. Samples were taken at 40, 50, 60, 70, and 80S. Each sample was analyzed for tritium and carbon-14 and then treated with $50 \mu\text{g}$ of pancreatic RNase per ml for 15 min at room temperature. All of the ^{14}C counts were solubilized by the RNase and lost during dialysis. They were then prepared for SDS-gel analysis. After electrophoresis, the total number of tritium counts in each maturation protein peak was corrected to 100% recovery and divided by the number of carbon-14 counts in RNA from the corresponding original gradient

fraction and the ratios from the five samples were compared. It was found that the ratios at 60, 70, and 80S were indeed constant (0.266, 0.242, 0.266, respectively), whereas those at 40 and 50S were increased by 73 and 27%, respectively. Since the labeled uracil was added before the onset of assembly, the RNA at 80S should have the same specific activity as the two previous intermediates even though the counts per minute in RNA at 80S is increasing greatly as a result of assembly.

That the ratio of maturation protein to RNA

FIG. 10. Infected cells were labeled with [^3H]leucine ($3 \mu\text{Ci/ml}$) from 18 to 29 min p.i. and with [^{14}C]uracil ($4 \mu\text{Ci/ml}$) from 15 to 29 min p.i. Samples were taken for lysis and gradient analysis at 21, 24, and 29 min p.i. The 80S regions were resedimented. (A) Counts per minute of tritium (protein) after: ●, 3-min label; Δ, 6-min label; ○, 11-min label. Symbols: ×, infectivity (plaque-forming units per milliliter); (B) counts per minute of carbon-14-labeled RNA after: ●, 6-min label; Δ, 9-min label; ○, 14-min label.

FIG. 11. Infected cells were labeled from 18 to 23 min p.i. with 10 μ Ci each of [3 H]leucine and [3 H]histidine per ml. The lysate was sedimented through a 6 to 20% sucrose gradient and the 70S region isolated. Half of the 70S sample was treated with 20 μ g of RNase per ml in TES. The other half was diluted equally with TES only. The two samples were resedimented and the 70 and 45S peaks were separately pooled and analyzed on SDS gels. (A) SDS-gel electropherogram of the resedimented, untreated, 70S sample. (B) SDS-gel electropherogram of the resedimented, RNase treated 70S sample; only the capsid peak (45S) was prepared for electrophoresis. The insets in both figures show the maturation protein on an expanded scale.

is the same at 80, 70, and 60S would be the expected result if they all contain the same relative amount of maturation protein. Since the 80S phage contains one molecule of maturation protein per particle (21) then the 60 and 70S structures must also contain only one.

DISCUSSION

The experiments reported here were designed to elucidate the various structures in which

MS2 RNA and protein can be found at late times after infection and to determine which structures might be involved in the assembly process. After sedimentation through sucrose gradients MS2 proteins are found at the top of the gradient in the soluble region, in a peak at 40S, in numerous structures sedimenting between 40 and 70S, and in 80S phage. The phage RNA is found in only the latter three regions. Continuous label and pulse-chase experiments

indicate that phage specific protein flows into and out of the soluble region first, and then, within a few minutes, into and out of the 40S peak and the assembly intermediates simultaneously, and, finally, into 80S phage.

The 40S peak contains MS2 RNA and all three phage proteins as shown also by Richelson and Nathans (14), and Cramer and Sinsheimer (1). Labeled coat protein and maturation protein contained in this peak can easily be chased into larger intermediates and whole phage, whereas the phage-specific RNA polymerase cannot. The last is not surprising since synthesis of the polymerase is stopped at about 20 min p.i. (24) and would, therefore, not be chaseable in these experiments. I feel that the peak examined in these studies is the same as that of Richelson and Nathans (14) and of Cramer and Sinsheimer (1). The latter authors detected a 40S peak at early times of infection (3 to 9 min p.i.) and a 45S peak at later times (after 18 min p.i.). This 45S peak, when resedimented through sucrose containing Tris-EDTA, was found to be composed of three components—45, 40, and 27S (free viral RNA). They hypothesized that the 40S peak is the polymerase-viral RNA complex and is probably identical to the peak found at early times of infection. Their 45S peak contains phage RNA, coat protein, and maturation protein. Label in the two proteins was found to decrease during a chase, indicating a viral precursor structure. No separation of 40 and 45S forms has been seen here even though the lysis procedure is the same as Cramer and Sinsheimer's and the gradients also contain Tris-EDTA (with the addition of 0.10 M NaCl). In fact, the properties of the 40S peak reported here are the same as the combined properties of the 40 and 45S peaks of Cramer and Sinsheimer; both species may well be present in this single, wide peak. The 40S peak, then, can consist of (i) a molecule of RNA with coat protein and maturation protein attached—the first assembly intermediate; and (ii) a complex of unknown composition between MS2 RNA and RNA polymerase. This is consistent with the pulse-chase results which show the coat protein and maturation protein to be chased from the 40S peak, whereas the RNA polymerase is not.

Assembly intermediates of size greater than 40S were identified by taking fractions between 40 and 80S from a primary sucrose gradient and resedimenting them through secondary sucrose gradients. These resedimentation experiments showed that from 40S to near 60S there are no intermediates which resediment as discrete peaks; rather broad, symmetrical peaks are

usually obtained upon resedimentation, indicating the possible presence of a great number of intermediates—too many to separate on sucrose gradients. In some experiments, samples taken from the 40 to 50S region gave two peaks after resedimentation, one near the original *S*-value (i.e., 43 or 50S), and another at about 20S (Fig. 4). The relative sizes of these two peaks varied from experiment to experiment, presumably because of partial digestion of the RNA contained in these intermediates by endogenous RNase. When assayed by SDS-gel electrophoresis, the composition of the peaks from the two samples was found to be quite dissimilar. The heavy peak (resedimenting at about 45S) from the 50S original sample contained all three phage proteins but with coat protein, maturation protein, and polymerase in the ratio 35:4:1 as judged by radioactivity contained in each. In contrast to this composition, the 20S peaks from both resedimented samples contained coat protein, maturation protein, and polymerase in the ratios 0.4:1:1 (50S original sample) and 0.6:1:1 (43S original sample). These ratios do not necessarily reflect the molar ratios of the three constituents; they are based on the counts per minute contained in each of the gel peaks. These results suggest that the assembly intermediates from 40S to at least 50S can be constructed in such a manner as to allow separation of a coat protein-rich segment (40 to 45S) from a maturation protein and polymerase-rich segment (20S) after limited degradation of the intermediate's RNA. Although this point has certainly not been proved, it is intriguing to speculate that the coat protein may bind to one segment of the RNA, whereas the maturation protein and polymerase may bind at another, separate, site. The presence of maturation protein bound in a restricted manner to the RNA of the first identifiable assembly intermediate can support the suggestion of Cramer and Sinsheimer (1) that maturation protein may need to be bound to the RNA in order to achieve a secondary structure which allows incorporation of the RNA into mature phage.

Samples taken from primary gradients between 80 and 60S display discrete peaks upon recentrifugation. There is a distinct peak at 70S and a somewhat less distinct peak at 60S. The 70S peak can be separated from both 80S phage and 60S intermediate, but the 60S peak always resediments with one shoulder at 70S and another on the light side. The resedimented 60S peak contains coat protein, maturation protein, and RNA polymerase. The 70S intermediate and 80S phage contain only coat protein and

maturation protein.

Since the ratio of counts in maturation protein to counts in RNA is the same for the 60S, 70S, and 80S structures, and the 80S phage contains only one molecule of maturation protein per particle (21), then the 70S and 60S intermediates must also contain only one, assuming an equal complement of RNA in all three structures. Calculations based on the data of Fig. 5 and other, similar experiments have indicated that the ratio of coat protein counts to maturation protein counts contained in the 80S phage peak is the same as the ratio in the 70S peak but approximately twice that in the 60S peak. The 80S and 70S peaks, then, contain the full 180-subunit complement of coat protein, but the 60S peak contains only about 90 subunits.

To determine whether the structure of any intermediate is dependent on protein-RNA interaction, samples taken from a primary gradient from 47 to 80S were treated with pancreatic RNase and recentrifuged. The sucrose gradient radioactivity profiles of the RNase-treated 47S, 53S, and 60S samples were similar—very little, if any, material appearing anywhere on the gradient except in the top few fractions. These results are consistent with the hypothesis that the structure of all intermediates from 40S to, and including, 60S depends on interaction between the coat protein and the RNA. The 70S and 83S samples, however, each exhibited two distinct peaks after RNase treatment. The RNase-treated 70S sample resulted in a peak at 70S and one at 45S; the 83S sample gave peaks at 80S and 45S. The 80S peak appearing after RNase treatment contains coat protein, maturation protein, and RNA, and is infectious; the 70S peak contains coat protein, maturation protein, and RNA, but is not infectious. The two 45S peaks contain coat protein and maturation protein, but no RNA. The 45S peak produced by RNase treatment of either 80S phage or 70S intermediate is considered to represent the complete phage capsid because it is RNase resistant, sediments at 45S, contains no RNA, and is composed solely of coat protein and maturation protein (in a ratio identical to 80S phage)—all properties of authentic capsid or “empty shell” (6, 7, 22).

The behavior of the RNase-sensitive and insensitive material resulting from the 70S and 80S peaks were examined during pulse-chase experiments. When samples were taken at 80S during the chase of a 3-min pulse (18 to 21 min p.i.), RNase treated, and recentrifuged to separate the 80S and 45S peaks, it was found that counts contained in the RNase-sensitive fraction (45S capsid) do not increase after about 3

min of chase, whereas those in the RNase-insensitive 80S phage are greatly increased throughout the chase. In a continuous label experiment the amount of RNase-sensitive material increased, again, for 6 min (18 to 24 min p.i.) and then remained constant. These two results, taken together, indicate that the synthesis of the 80S RNase-sensitive form is stopped at about 24 min p.i. This RNase-sensitive form may be the same as the noninfectious 80S particle found early in MS2 infection by Godson (3).

Similar experiments were performed on the 70S intermediate but the results were much more difficult to interpret. During continuous protein label starting at 18 min p.i., radioactivity increased rapidly at 70S for 6 min and then more slowly for at least an additional 5 min. During the chase of an 18 to 21 min p.i. pulse it was found that protein label at 70S did not decrease but actually increased somewhat, suggesting 70S to be a product rather than an intermediate. When samples were taken at 70S during a chase, treated with RNase and resedimented, it was found that radioactivity contained in the RNase-sensitive form decreased, whereas that in the insensitive form increased for at least the first 8 min of chase. Most of the change occurred during the first 4 min of chase. This behavior of the RNase-insensitive 70S structure is indicative of a product rather than an intermediate of assembly. The identity of the RNase-sensitive 70S structure is more difficult to ascertain. It is not the same as the “defective particle” of Steitz (21) because it contains the maturation protein. Since protein radioactivity contained in it can be chased out, it is most likely an assembly intermediate; however, from the present data, it cannot be determined whether it is an intermediate in the assembly of 80S phage or of 70S RNase-insensitive particles. If the 70S RNase-sensitive particle is, in fact, a precursor to the 70S RNase-insensitive particles, then a change in the arrangement of the capsid constituents, resulting in a structure which can fully protect the RNA from nuclease degradation, could effect the change from RNase sensitivity to insensitivity. This type of structural alteration would, of necessity, occur in the absence of further coat protein addition since a presumably complete capsid is present in both the RNase-sensitive and insensitive particles. A change from a relatively loose to a more compact structure might be expected to increase the sedimentation rate of the particle, an effect which is not observed. If, however, the RNase-sensitive particle is an intermediate in phage assembly, then a structural change without addition of coat protein could easily result in

a phage particle of 80S.

In conclusion, I would like to propose a model of MS2 assembly consistent with the in vivo results presented here. Assembly begins with the appearance of a 40S structure containing RNA, coat protein, maturation protein, and phage RNA polymerase. Most of the coat protein is found on one segment of the RNA and most of the maturation protein is found on another segment after partial disruption of the 40S structure by endogenous ribonuclease activity, indicating nonrandom binding of the three proteins. Assembly progresses by addition of coat protein to the 40S intermediate, resulting in a large number of indistinct intermediates from 40 to 60S, and one distinct intermediate at 60S. These intermediates contain an increasing amount of coat protein relative to maturation protein, polymerase, and RNA (Fig. 5). Addition of large amounts of pancreatic RNase destroyed practically all structure associated with the intermediates. Thus, in the absence of RNA, the partially assembled capsid is quite unstable.

Addition of coat protein to the 60S intermediate results in a 70S structure consisting of RNase-sensitive and insensitive portions. The RNase-insensitive fraction is not an assembly intermediate, whereas the RNase-sensitive fraction probably is. The RNase-sensitive 70S structure contains the complete capsid as evidenced by the 45S peak resulting from RNase digestion and by a ratio of coat protein:maturation protein:RNA identical to that of 80S phage particles. A structural change of the 70S intermediate resulting in the formation of 80S mature phage is proposed as the last step in assembly.

An additional factor to be considered in a discussion of MS2 assembly is the presence of the phage RNA polymerase in all intermediates from 40 to 60S but not in the 70S structures or the 80S phage. Although it is quite possible that the polymerase is actually a part of numerous RNA replication complexes which cosediment with the assembly intermediates, it is also possible that it is a requisite component of assembly. The amount of polymerase in resedimented samples, as judged by radioactivity appearing on SDS-gels, is nearly the same as the amount of maturation protein contained in the same intermediates. Each intermediate might, then, contain one molecule of polymerase per molecule of maturation protein which is removed by the time of capsid completion (the 70S structures). This could imply that phage RNA polymerase is cycling between RNA synthesis and assembly.

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