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Process of Infection with Bacteriophage φX174

XXXVII. RNA Metabolism in φX174-Infected Cells

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The RNA produced in vivo from bacteriophage φX174 DNA has been analyzed by polyacrylamide-agarose gel electrophoresis and sedimentation in dimethyl sulfoxide gradients, and the results of Hayashi and Hayashi (1970) have been confirmed and extended. An efficient procedure for recovery of RNA from gels, followed by a hybridization assay, has indicated the presence in infected cells of 18 distinct RNA species with sizes up to and greater than the unit (viral) length. The sizes of φX mRNA's were similar irrespective of whether material was analyzed on gels or in dimethyl sulfoxide gradients. When virus-induced RNA was detected by a double-label method, seven additional low-molecular-weight species were observed on gels and the resolution of dimethyl sulfoxide gradients was enhanced. The present results lend support to aspects of the model of Hayashi and Hayashi (1970) for the generation of these discrete mRNA species; an alternative model is also discussed.

The infective process of the single-stranded DNA bacteriophage φX174 has been studied extensively in terms of viral DNA replication (22, 24, 37) and synthesis of virus-specific proteins (7, 12).

Considerable work has also been carried out on the metabolism of φX mRNA. Early observations demonstrated that the in vivo φX mRNA was transcribed off the RF strand complementary to the phage (+) strand (19), and a pulse-labeled RNA-RF hybrid was reported (17). More recent studies on the size and number of φX mRNA species have yielded various conclusions. Hayashi and Hayashi (20) analyzed φX mRNA by polyacrylamide gel electrophoresis and identified 10 discrete RNA species with molecular weights ranging from 3 $\times 10^6$ (almost two complete rounds of transcription) to 0.2×10^6. Puga and Tessman (31) found discrete mRNA species for the related phage S13. In contrast, the studies of Sedat and Sinsheimer (34), in which RNA was completely denatured by sedimentation in the presence of dimethyl sulfoxide (Me_2SO), showed a broad heterogenous size distribution with a mean RNA mol wt of 3×10^5 and an upper limit of 1.6×10^6 to 1.7×10^6 (unit-length RNA).

Hayashi and Hayashi (20) also observed that most of these discrete mRNA species are stable and there was no apparent precursor product relationship among these RNAs. Based on these observations, Hayashi and Hayashi postulated the following. (i) Since some of the RNAs that are smaller than the entire genome length have molecular weights higher than any known φX174 cistron size, these must be polycistronic. (ii) When the molecular weights of the RNA peaks smaller than full genome size are added, the sum is far in excess of the molecular weight of the mRNA expected from the entire genome. Therefore, mRNA of a particular cistron must exist in more than one peak. (iii) The RNAs that have molecular weights larger than the full genome length must be the result of more than one round of transcription of RF DNA.

It was of interest to obtain further information on the size and numbers of in vivo RNA species and to correlate observations made by using denaturing and nondenaturing systems of analysis. In addition, the presence of phage proteins in very different molar amounts through infection (30) suggested that control of synthesis might be exerted at the level of RNA synthesis. Information on the nature of in vivo species is also important in that it seems likely that the ready availability and small size of the φX DNA template will allow a direct comparison between these RNAs and material made in vitro.

In the present study, φX mRNA pulse-labeled for various times with different isotopes was analyzed on acrylamide-agarose gels and Me_2SO gradients. Virus-induced RNA was de-

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tected by hybridization to ϕX RF and by a double-label method which compared pulses of similar duration in infected and mock-infected cells.

MATERIALS AND METHODS

Media. The medium used was TPA, which is TPG (38) containing a mixture (2.7 g/liter) of 20 natural *l*-amino acids (Nutritional Biochemicals Corp.). TPA was supplemented with thymine (7 μg/ml). TPA-low phosphate contained 0.1 the normal phosphate concentration. KC broth, bottom agar, and top agar for phage plating were as described previously (36).

Phage and bacteria. ϕX*am*3, a lysis-defective amber mutant (21), was a gift from R. G. Rohwer. Stocks, obtained from a single plaque and purified by cesium chloride banding followed by sucrose density gradient centrifugation, had a PFU/particle ratio of 0.13. Phage titer was assayed on *Escherichia coli* HF4714, a permissive host for ϕX amber mutants. *E. coli* HF4704 (*thy*$^-$,*hcr*$^-$,*su*$^-$), described by Lindqvist and Sinsheimer (26), was the nonpermissive host strain.

Materials. [5-^{3}H]uracil (specific activity, 19 Ci/mmol) and [2-^{14}C]uracil (specific activity, 60 mCi/mmol) were obtained from Schwarz/Mann. ^{32}P- and ^{33}P-labeled phosphoric acid (both carrier free) were obtained from New England Nuclear. Spectroquality Me_2SO and formamide were purchased from Matheson, Coleman and Bell. Deuterated (d_6) Me_2SO, acrylamide, *N,N*^{1}-methylene bisacrylamide, *N,N,N*^{1},*N*^{1}-tetramethylethylenediamine, and ammonium persulfate were all from Bio-Rad Laboratories. Agarose, pancreatic RNase, and egg white lysozyme (crystallized three times) were supplied by Sigma Ltd. DNase 1 (RNase free) was obtained from Worthington Biochemicals, and batches were checked by incubation with *E. coli* rRNA and sedimentation of products on Me_2SO gradients. (Several lots had significant RNase activity.) Sodium dodecyl sulfate (especially pure) was obtained from British Drug Houses Ltd. Phenol from Mallinckrodt was redistilled and stored at −20 C under nitrogen.

Labeling and extraction of ϕX mRNA. Cells were grown with aeration to 5 × 10^8 cells/ml in 20 ml of TPA (labeling with uracil) or TPA-low phosphate (labeling with phosphate) and then were infected at a multiplicity of 4. Twenty minutes after infection, cultures were pulsed with [5-^{3}H]- or [2-^{14}C]uracil, usually at 30 to 50 μCi/ml, or with $H_3{}^{32}PO_4$ or $H_3{}^{33}PO_4$, at 100 μCi/ml. Pulses were terminated by addition of a sodium azide (0.5 M)-potassium cyanide (0.03 M) mixture to final concentrations of 0.05 and 0.003 M, respectively. Each culture was immediately poured into a 500-ml flask precooled in a dry ice-methanol bath. Cultures were completely frozen within 12 s.

Cultures were thawed and cell pellets, obtained by centrifugation in the cold, were suspended in 6 ml of Tris-NaCl-EDTA buffer (0.05 M Tris, 0.1 M NaCl, 0.01 M EDTA, pH 7.5); lysozyme (2 mg/ml in Tris-NaCl-EDTA) was added to 0.2 mg/ml, and the suspensions were maintained at 22 C for 3 min. The volume was made 10 ml with Tris-NaCl-EDTA, and sodium dodecyl sulfate (10%, wt/vol) was added to 0.5%. Lysates were extracted twice at 22 C with an equal volume of phenol that had just previously been equilibrated with Tris-NaCl-EDTA plus 0.5% sodium dodecyl sulfate. RNA was precipitated from the final aqueous phase by addition of 3 volumes of 95% ethanol and storage at −20 C overnight. Ethanol precipitates, collected by centrifugation at 6,000 × *g* for 10 min at 0 C, were reprecipitated from 10 ml of Tris-NaCl-EDTA plus 0.1% sodium dodecyl sulfate. Precipitates were suspended in 1 ml of 0.1 M sodium acetate-2.5 mM magnesium sulfate (pH 5.0) containing 25 μg of DNase I. After incubation at 22 C for 30 min, the volume was made 10 ml with Tris-NaCl-EDTA plus 0.1% sodium dodecyl sulfate, and one phenol extraction was performed. RNA was reprecipitated from 10 ml of Tris-NaCl-EDTA and samples were stored in ethanol at −20 C.

Baby hamster kidney (BHK-21) cell RNA and Sindbis-specific RNA were labeled and isolated as described by Simmons and Strauss (35). *E. coli* rRNA was prepared by the method of Godson and Sinsheimer (16).

DNA-RNA hybridization procedures. ϕX*am*3 closed circular duplex DNA (RFI), isolated by the method of Komano and Sinsheimer (25), was purified by centrifugation in a cesium chloride equilibrium gradient in the presence of ethidium bromide (2). RFI was more than 95% pure as judged by alkaline sedimentation in a model E ultracentrifuge. Nitrocellulose filters, disks of 11-mm diameter (each containing 9 to 10 μg of RF), and blank filters were prepared as described by Truffaut and Sinsheimer (43).

Hybridization in formamide was as described by Gillespie and Gillespie (13). Polyacrylamide-agarose gel slices were agitated in a horizontal shaker in vials containing 0.4 ml of 50% (vol/vol) formamide-6 × SSC (1 × SSC = 0.15 M NaCl plus 0.015 M sodium citrate) (pH 7.0) at 37 C for 48 h. Under these conditions, good and reproducible RNA recoveries were obtained when, as a control, gels were polymerized in the presence of the total RNA isolated from ϕX-infected cells, and the presence of the gel slice did not affect the efficiency of hybridization. After elution, a 20-μliter sample was taken for assay of radioactivity, and then a blank filter and, subsequently, an RF filter were added. Vials were capped tightly and incubated at 37 C for 30 h with occasional shaking. After incubation, filters were washed twice by vortexing in 10 ml of 2 × SSC and then were treated with 1 ml of pancreatic RNase (25 μg/ml in 2 × SSC, pH 5.0, preheated to 84 C for 10 min to eliminate contaminating DNase) for 40 min at room temperature. After two more washes with 2 × SSC, filters were dried and counted in a toluene-based scintillation fluid. Counts on blank filters were subtracted from values of the appropriate RF filters. Blank values ranged from 0.001 to 0.05% of the hybridized counts.

Hybridization in Me_2SO was as described by Sedat and Sinsheimer (34). An Me_2SO-SSC (pH 7.0) mixture was added to a final volume of 0.4 ml, and final concentrations of 40% (vol/vol) Me_2SO-2 × SSC (pH

7.0) vials were incubated at 37 C for 50 h and then processed as before.

Hybridizations in 6 × SSC were performed by the method of Gillespie and Spiegelman (14). RNA precipitates were dissolved in 0.4 ml of 6 × SSC, and vials were incubated at 66 C for 30 h with occasional shaking.

Electrophoresis. Composite gels (2.0% acrylamide, 0.4% agarose) were made as described previously (8). RNA samples (25 to 50 μg) in 25 μliters of stock buffer containing 15% (wt/vol) sucrose were applied directly to the gel surface. Electrophoresis was continued at 6 mA/gel for 3 to 4 h at 4 C. Gels were frozen and cut immediately into 1-mm segments with a Mickle gel slicer (Brinkman Instruments Ltd.). For determination of radioactivity, slices in scintillation vials were shaken overnight in darkness with 6 ml of 90% (vol/vol) toluene fluor–9% (vol/vol) Nuclear-Chicago solubilizer–1% (vol/vol) distilled water.

Me_2SO gradients. Deuterated Me_2SO (33) was used in the gradients. RNA (in 10 μliters of 0.01 M Tris-hydrochloride, pH 7.8, containing less than 100 μg of RNA) plus 100 μliters of Me_2SO and 10 μliters of dimethyl formamide was layered on top of the gradient. Gradients were centrifuged at 64,000 rpm for 11 h at 27 C in a Spinco SW65 rotor or at 50,000 rpm for 14 h in an SW50.1 rotor.

Samples of collected fractions spotted onto glass-fiber (GF/A) disks were heated to 100 C for 90 min and then counted using a toluene-based scintillation fluid.

Detection of virus-induced RNA by a double-label method. Infection with φX174 has little effect upon the amount of host RNA or protein synthesis (26) (except at high multiplicities of infection, where changes in membrane permeability result in decreased host synthesis [40]). This circumstance allows virus-induced RNA, which could consist of both virus-specific and host-specific components, to be identified by the double-label method previously used to detect φX-induced proteins (3, 30). In these experiments ^{32}P was used to label infected cells and ^{33}P was used to label mock-infected cells for similar pulse times. Pulses were terminated as before and cells were pelleted separately. Infected and mock-infected cells were each suspended in 3 ml of Tris-NaCl-EDTA. These cell pellets were combined and RNA was isolated as described previously. The amount of φX-induced ^{32}P counts in a fraction ($\Delta^{32}P$) can be determined according to the equation $\Delta^{32}P = {}^{32}P - {}^{33}P$ (R), where ^{32}P and ^{33}P are the corrected counts per minute in the fraction and R is the $^{32}P/^{33}P$ ratio from a fraction presumed to contain no φX-induced material.

Radioactivity measurements. Radioactivity was measured with a Beckman LS-233 liquid scintillation counter. For double- and triple-label separations, background subtractions, channel spillovers and, where appropriate, ratio and Δ calculations were computed by using programs for an IBM 370/155 computer developed by R. G. Rohwer and J. R. Bell, Division of Biology, California Institute of Technology. Corrected count rates, percent total counts per minute, ratios, ratio order indexes, Δ and percent total Δ, with appropriate standard deviations, could be calculated. For any one data set, up to three calculated parameters could be plotted simultaneously with a Calcomp plotter. Care was taken in preparation of spill standards. For example, after each gel run, standard gels polymerized in the presence of high-molecular-weight RNA labeled with the appropriate isotope were sliced, and the RNA then solubilized in the usual way.

RESULTS

Size and number of φX-specific RNAs as determined by hybridization. The effects of varying pulse duration, together with comparisons between ^{32}P and [^{3}H]uracil labeling of φX mRNA, were investigated. Figure 1 shows the effect of increasing pulse time on hybridizable RNA (expressed as a percentage of total labeled RNA) and on the specific activity of the total labeled RNA. With ^{32}P labeling (Fig. 1b), the proportion of hybridizable counts increased for some 2 min to a value of 14 to 15% and then remained at a constant level. In contrast, the proportion of hybridizable RNA remained constant at approximately 10 to 12% for pulses of [^{3}H]uracil (Fig. 1a) from 10 s to 10 min. This slower equilibration of the labeled phosphate

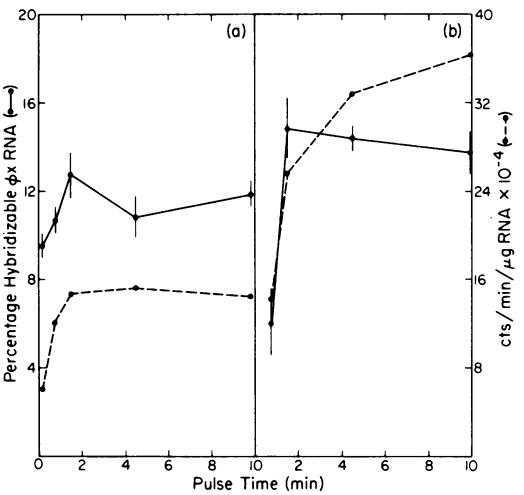

FIG. 1. *Effects of increasing pulse time on the proportion of φX mRNA and on the specific activity of total labeled RNA. Cells infected with φXam3 at a multiplicity of 4 were pulse-labeled with [^{3}H]uracil (a) or ^{32}P (b) 20 min after infection, and the RNA was isolated. The amount of φX mRNA was estimated by hybridization to RF DNA in 6 × SSC at 66 C for 30 h. (——) φX hybridizable RNA expressed as a percentage of total labeled RNA. Each point represents the mean of five separate estimations, and 95% confidence limits for the standard errors of means are indicated. (-----) Specific activity of total labeled RNA.*

pools also was reflected in the increase in specific activity of total labeled RNA. These results suggest that the viral RNA is made from a different pool of precursors than is the cellular RNA.

Pulse-labeled RNA was analyzed further under both denaturing and nondenaturing conditions. Single- and double-stranded RNAs are completely denatured in Me_2SO (41). Simmons and Strauss (35) established that gradients containing deuterated (d_6) Me_2SO could be used to determine RNA molecular weight. They showed that a plot of the logarithm of RNA molecular weight against the logarithm of distance traveled was linear. This procedure for RNA size estimation has an advantage over procedures such as gel electrophoresis since conformational effects are eliminated. In the present study, the relationship determined by Simmons and Strauss was confirmed by using a double-labeled mixture of BHK and *E. coli* rRNA markers.

RNA isolated from infected cells pulse-labeled with ^{32}P for 45 s, 90 s, 5 min, and 10 min was co-sedimented with ^{3}H-labeled *E. coli* rRNA (Fig. 2a and b). With increasing pulse

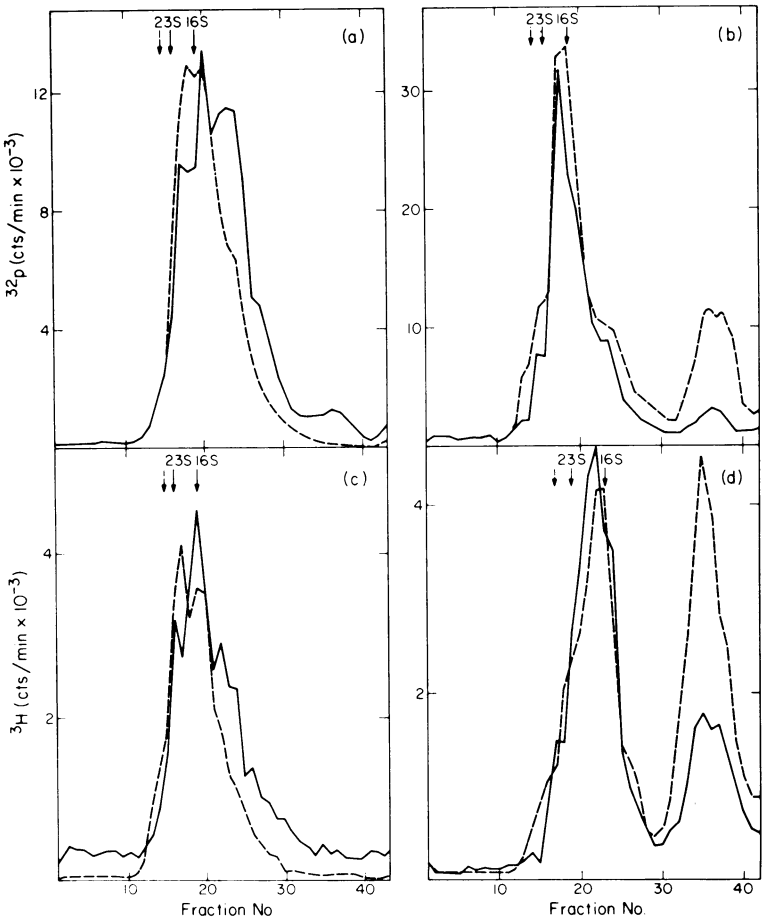

FIG. 2. *Effect of labeling with different isotopes (in different precursors) and of varying the pulse time on the size of φX mRNA as determined by sedimentation in Me_2SO gradients. Cells, infected with φXam3, were pulse-labeled with [^{3}H]uracil or ^{32}P; RNA was isolated as described. Pulse-labeled RNA was co-sedimented with the appropriate ^{32}P- or ^{3}H-labeled E. coli rRNA marker. After a 10-μliter volume of each collected fraction was counted, φX mRNA was located by hybridization to RF DNA in 40% (vol/vol) Me_2SO-2 × SSC (pH 7.0) at 37 C for 50 h. (a) Cells labeled with ^{32}P for 45 and 90 s; (b) cells labeled with ^{32}P for 5 and 10 min; (c) cells labeled with [^{3}H]uracil for 10 and 45 s; (d) cells labeled with [^{3}H]uracil for 5 and 10 min. (——) Radioactivity profile of hybrid counts for the shorter labeling time in each pair of labeling times. (-----) Radioactivity profile of hybrid counts for the longer labeling time in each pair of labeling times. Arrows show the positions of 16S and 23S E. coli rRNA markers, and the point on each gradient equivalent to unit-length φX mRNA is indicated by the dashed arrow. Sedimentation is from right to left.*

time, the mean molecular weight of hybridizable RNA increased from some 250,000 at 45 s to a maximum of 700,000 at 5 min. Such a marked increase in molecular weight with pulse time cannot be observed when labeling with [^{3}H]uracil. Here, RNA pulse-labeled with [^{3}H]uracil for 10 s, 45 s, 5 min, and 10 min was co-sedimented with ^{32}P-labeled *E. coli* rRNA (Fig. 2c and d). Even a 10-s pulse was sufficient to label RNA with a mean size of 500,000 daltons, although an equilibrium pattern and maximal size were not attained until a 45-s pulse was used. A 90-s uracil pulse produced the same pattern of labeled RNA as the 45-s pulse.

At longer pulse times, with [^{3}H]uracil labeling, and to a lesser extent with ^{32}P labeling, hybridizable counts appeared at the top of the gradients (30,000 mol wt and less), indicating some RNA breakdown; the *S* of maximal radioactivity also shifted slightly toward a lower value (Fig. 2c and d). Peaks and shoulders were apparent on the radioactivity profiles for all of the pulse times, and at longer times a discrete shoulder was observed at the position of unit-length φX mRNA. The proportion of radioactivity present in material of greater than unit length was small, comprising 2 to 3% of the total hybridizable counts per gradient.

Although Me_2SO gradients are useful for analysis of RNA in the absence of configurational restraints, their resolution is poor compared to that provided by polyacrylamide gels. Electrophoresis of RNA through polyacrylamide gels can provide a reasonably accurate method of determining RNA molecular weight if appropriate markers are available (4). Empirically, the distance of RNA migration was shown to vary as the logarithm of molecular weight. In this study, the molecular weights of marker RNA species were taken to be 0.56 and 1.07×10^6 for *E. coli* rRNA (39), 0.71 and 1.90×10^6 for BHK rRNA (28), and 1.60 and 4.0×10^6 for Sindbis 26*S* and 49*S* RNA (35).

RNA from φX-infected cells labeled with ^{32}P for 45 s, 90 s, and 5 min was co-electrophoresed with ^{3}H-labeled BHK and ^{3}H-labeled *E. coli* rRNA's on 2% polyacrylamide-agarose gels. Profiles of hybridizable counts across the gels are shown in Fig. 3a, b, and c.

Similarly, RNA pulse-labeled with [^{3}H]uracil for 45 s, 5 min, and 10 min was co-electrophoresed with ^{32}P-labeled Sindbis-specific and ^{33}P-labeled *E. coli* rRNA markers (Fig. 3d, e and f). In both ^{32}P- and ^{3}H-labeled samples, discrete size classes were apparent even at the shorter pulse times. The molecular weights of these species were highly reproducible, and values did not vary appreciably with pulse duration. However, the relative proportions of individual species did vary with pulse duration; this was especially noticeable in short pulses with ^{32}P labeling, because of the slower equilibration of this label. At shorter pulse times, the distribution of radioactivity was weighted towards the lower-molecular-weight species (Fig. 3a). This situation was reversed at longer times (Fig. 3c) when a stable pattern was generated.

Separation of the individual species is most clearly shown in Fig. 3d, where, in contrast to the ^{32}P-labeling profile, a 45-s [^{3}H]uracil pulse labeled what was approximately a final stable pattern. The molecular weights of these species (Fig. 3d), together with three additional species that were better resolved on other gels, are listed in Table 1. Frequencies of occurrence of each species based on other gel profiles also are recorded. There were 15 major φX mRNA species of unit length (26*S*) and smaller, with three obvious peaks greater than unit length. The relative proportions of the species appeared to vary somewhat with pulse length. At longer pulse times, hybridizable counts accumulated in some low-molecular-weight material (Fig. 3f), although the same stable pattern of larger species was observed.

It is apparent that these RNA size changes associated with pulse times are comparable to the differences detected on Me_2SO gradients.

Identification of virus-induced RNAs by a double-label method. Data for a 5-min pulse with mock-infected cells only is shown in Fig. 4a and b. In these controls, the ^{32}P/^{33}P ratio was constant across Me_2SO gradients and polyacrylamide-agarose gels, and this result was independent of pulse time. Ratios at the beginning of gels and gradients fluctuated somewhat since counts were low in these regions. Controls demonstrated that isotope reversal had no effect upon virus-induced ratio changes.

The effect of φX infection on ^{32}P/^{33}P ratios across polyacrylamide-agarose gels was determined. Ratio changes and Δ^{32}P profiles, calculated with ratios from the fractions indicated, for 90-s and 5-min pulses are shown in Fig. 5a and b. Discrete virus-induced ratio and Δ^{32}P changes were observed for both pulse times, and the molecular weights of Δ^{32}P species for the 90-s pulse are listed (Table 2a) together with their frequency of occurrence on gels not shown here.

The molecular weights of these induced species correlate with values obtained by the hybridization technique (Table 1), although the resolution of higher-molecular-weight RNAs (0.26 to 3.4×10^6) was slightly less than that afforded by hybridization (Fig. 3b). However, in

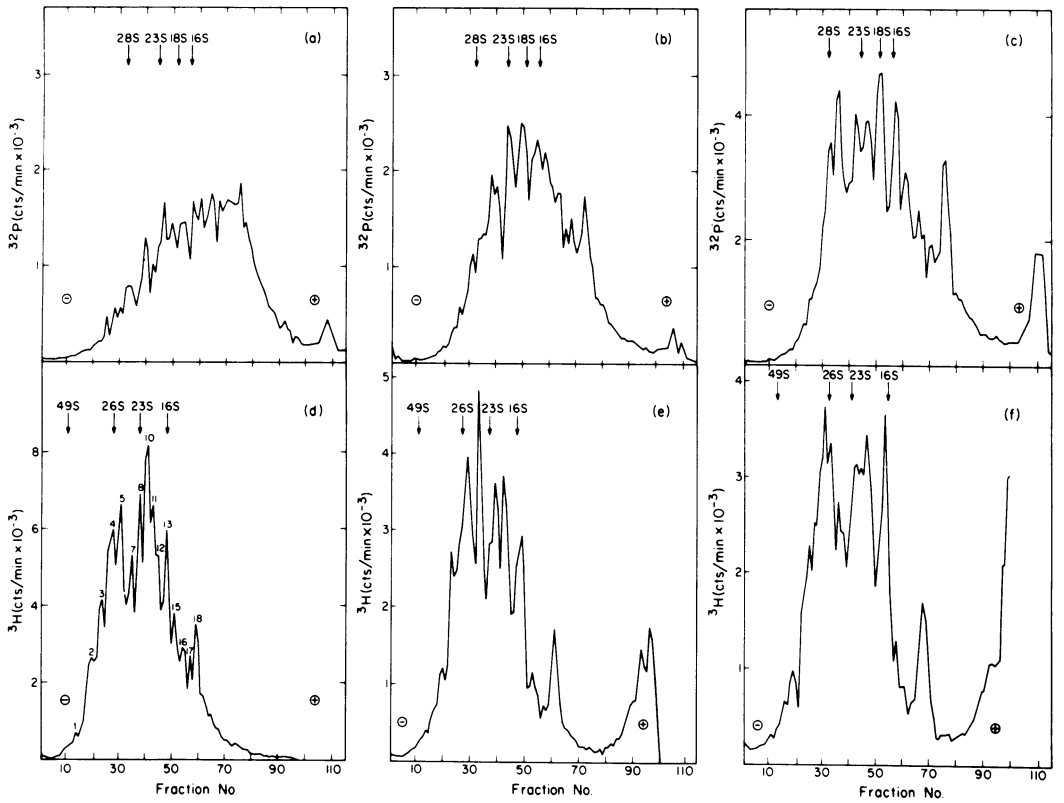

FIG. 3. *Polyacrylamide-agarose gel electrophoresis of φX mRNA labeled with [^{3}H]uracil or ^{32}P for different pulse durations. Pulse-labeled RNA was co-electrophoresed with ^{3}H-labeled E. coli and BHK rRNA's or with ^{32}P-labeled E. coli rRNA and Sindbis-specific RNA. RNA was recovered from gel slices by being shaken in vials containing 0.4 ml of 50% (vol/vol) formamide-6 × SSC (pH 7.0) at 37 C for 48 h. After elution, a 20-μliter sample was removed to locate the marker RNAs; blank and RF filters were then added directly to vials, and φX mRNA was detected by hybridization at 37 C for 30 h. Cells were labeled with ^{32}P for (a) 45 s; (b) 90 s; and (c) 5 min. Cells were labeled with ^{3}H for (d) 45 s; (e) 5 min; and (f) 10 min. (——) Radioactivity profile of ^{32}P- and ^{3}H-hybridizable counts. Arrows show the position of 16S and 23S E. coli rRNA, 18S and 28S BHK rRNA, and 26S and 49S Sindbis-specific RNA. Electrophoresis is from left to right. Peak numbers of Fig. 3d refer to the RNA species listed in Table 1.*

the lower-molecular-weight region (0.19 to 0.07 × 10^{6}), seven minor Δ^{32}P species were resolved by the double-label method. These minor species were apparent only as slight shoulders on the hybridizable profile. The Δ^{32}P profile for a 5-min pulse differed somewhat from the 90-s profile, with large peaks at 23S and just slower than 16S. These two peaks, which were not obvious as ratio changes, were generated by high count rates in the regions of host rRNA. Further observations on the nature of these two peaks will be outlined in a subsequent publication. Minor low-molecular-weight species were also detected at 5 min. Again, these were apparent as shoulders on the hybridizable profile (Fig. 3c).

Ratio and Δ^{32}P profiles across Me_2SO gradients for 45- and 90-s pulses are shown in Fig. 5c and d. From ratio changes there was evidence for nine discrete species, and their molecular weights are listed in Table 2(b). The Δ^{32}P profiles resembled the hybridizable profiles (Fig. 2a) and showed a mean size increase with pulse time.

DISCUSSION

Detection of φX mRNA's. We have confirmed and extended the results of Hayashi and Hayashi (20) (as well as the results of Puga and Tessman [31] for S13 mRNA) indicating the presence of multiple φX mRNA's in φX174-

TABLE 1. *Molecular weights of φX174 mRNA's as determined by hybridization*[a]

RNA species	Mol wt (×10⁶)
1	3.42 ± 0.11 (7)
2	2.45 ± 0.09 (8)
3	1.99 ± 0.04 (8)
4	1.60 ± 0.06 (8)
5	1.35 ± 0.04 (6)
6[b]	1.29 ± 0.03 (4)
7	1.10 ± 0.06 (6)
8	1.07 ± 0.04 (7)
9[b]	0.93 ± 0.03 (4)
10	0.77 ± 0.05 (6)
11	0.70 ± 0.02 (3)
12	0.63 ± 0.02 (5)
13	0.56 ± 0.05 (6)
14[b]	0.50 ± 0.03 (4)
15	0.45 ± 0.02 (8)
16	0.38 ± 0.04 (7)
17	0.32 ± 0.02 (7)
18	0.28 ± 0.02 (8)

[a] Values are the molecular weights of species indicated in Fig. 3d. The values in parentheses show the number of gels in which each species has been observed. Errors represent extreme fluctuations from these values in a total of eight gels examined.

[b] These are three additional species that were resolved on other gels.

infected cells. The high resolution of polyacrylamide-agarose gel electrophoresis, together with an efficient procedure for recovery of RNA and hybridization across gels, has enabled us to detect 15 distinct RNA species of unit length and less with three obvious components greater than unit length. It was not possible to obtain accurate estimates of relative molar amounts due to the number of species detected.

With increasing pulse time, no obvious precursor-product relationships among the RNAs were observed, in agreement with the results of Hayashi and Hayashi (20). Detection of virus-induced RNA by a double-label method enabled seven additional low-molecular-weight species to be detected on gels and greatly enhanced the resolution achieved with Me_2SO gradients (Fig. 5c and d).

No evidence was obtained for specific effects of virus infection on host mRNA's. This double-label technique may be of some general use in detecting virus-induced RNAs especially under conditions where host synthesis is not inhibited by infection. The procedure obviates the use of inhibitors and other methods designed to reduce host RNA synthesis.

Molecular weight values (Tables 1 and 2) are in general agreement with values for the 10 φX mRNA's detected by Hayashi and Hayashi (20)

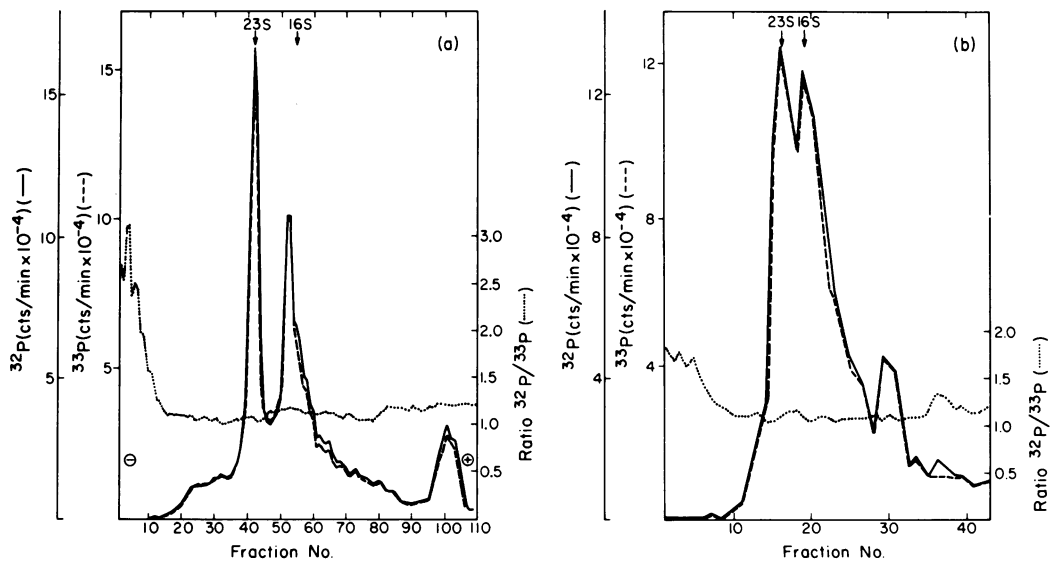

FIG. 4. *Controls for identification of virus-induced RNA by a double-label method. A mock-infected cell culture was pulse-labeled with ^{32}P for 5 min, and a similar culture was pulsed with ^{33}P for 5 min. The cells were spun down separately, cell pellets were combined, and RNA was isolated. The double-labeled RNA mixture was analyzed in the presence of ^{3}H-labeled E. coli rRNA marker. (a) Electrophoresis on a polyacrylamide-agarose gel. (b) Sedimentation in a Me_2SO gradient. (——) ^{32}P radioactivity profile. (-----) ^{33}P radioactivity profile. (·····) Ratio of $^{32}P/^{33}P$ radioactivity. Arrows show the positions of 16S and 23S E. coli rRNA's.*

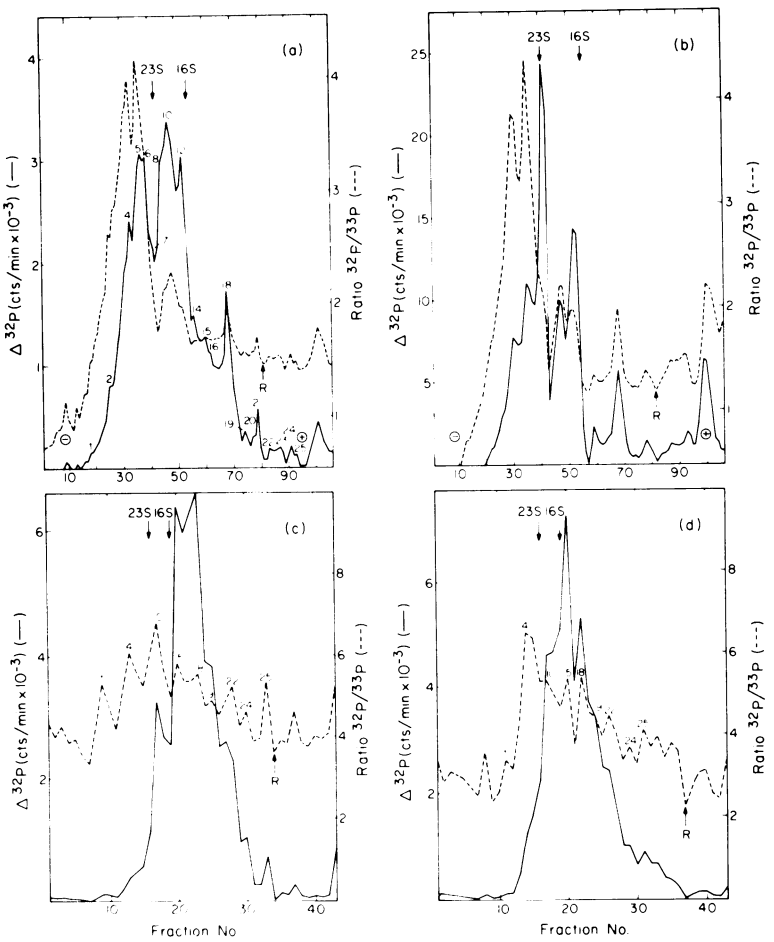

FIG. 5. *Identification of φX-induced RNA by a double-label method. An infected cell culture was pulse-labeled for various times with ^{32}P, and a mock-infected culture was pulsed for a similar time with ^{33}P. Cell pellets were combined and RNA was isolated. The double-labeled mixture of RNAs was analyzed in the presence of ^{3}H-labeled E. coli rRNA marker. For each fraction, a $^{32}P/^{33}P$ ratio was calculated and $\Delta^{32}P$ was computed using the ratio from the indicated fraction. (a) Polyacrylamide-agarose gel of RNA from cells labeled for 90 s; (b) polyacrylamide-agarose gel of RNA from cells labeled for 5 min; (c) Me_2SO gradient of RNA from cells labeled for 45 s; (d) Me_2SO gradient of RNA from cells labeled for 90 s. (——) $\Delta^{32}P$ radioactivity profile. (-----) Ratio of $^{32}P/^{33}P$ radioactivity. Peak numbers in (a), (c), and (d) refer to the RNA species listed in Table 2.*

and with the eight S13-specific RNAs observed by Puga and Tessman (31). The S13 mRNA's were obtained from polysome fractions, implying that they are functional in translation. φX mRNA sizes obtained by gel electrophoresis and sedimentation in Me_2SO gradients were in good agreement, and there was no evidence for RNA aggregates on gels such as detected by Jacob et al. (23) in M13-infected cells.

Stability of φX mRNA's. The slower equilibration of the phosphate label, compared to that of uracil (Fig. 1), which is known to equilibrate almost immediately (27), effectively increased the apparent time required for synthesis of discrete RNAs. This was observed on both gels and Me_2SO gradients at shorter pulse times and provides an explanation for the somewhat lower RNA sizes obtained by Sedat and Sinsheimer (34), who used a short (45 s) pulse of ^{32}P which was insufficient to label the stable RNA pattern (Fig. 3a and b). Detection of discrete RNA species with long label times (5 and 10 min), when molecules were in various stages of synthesis at short label times, implies that the half-life is at least equal to the time of synthesis. Assuming an in vivo synthesis rate of

TABLE 2. *Molecular weights of φX mRNA's as determined by a double-label method*

RNA species	Mol wt[a] (×10⁶)	
	a	b
1	3.38 (6)	3.26 (5)
2	2.50 (8)	
3		
4	1.66 (7)	1.66 (5)
5	1.34 (5)	
6	1.23 (5)	
7	1.09 (7)	
8	1.02 (4)	
9		
10	0.80 (8)	0.80 (6)
11[b]	0.70 (3)	
12	0.63 (4)	
13		
14	0.50 (6)	
15	0.44 (7)	0.45 (6)
16	0.37 (7)	
17[b]	0.32 (3)	
18	0.26 (7)	0.28 (6)
19	0.19 (6)	0.18 (6)
20	0.17 (8)	
21	0.15 (7)	
22	0.12 (5)	0.12 (4)
23	0.10 (7)	
24	0.08 (7)	0.08 (6)
25	0.07 (7)	0.07 (6)

[a] (a) Molecular weights of species associated with ratio changes across a polyacrylamide gel (Fig. 5a). (b) Molecular weights of species associated with ratio changes across a Me_2SO gradient (Fig. 5c). Figures in parentheses show the number of occurrences of each species in a total of eight gels and six gradients examined.

[b] These are two additional species that were resolved in other gels.

1,000 to 1,500 nucleotides per min (1, 5, 22), with immediate equilibration of label, completion of unit-length φX mRNA (5,500 nucleotides) would take 4 to 5 min. Hayashi and Hayashi (18, 20) have reported that individual φX mRNA species were stable for at least 10 min after a chase with cold uridine and M13 mRNA has a half-life of 20 min (23). It appears that greater stability of phage mRNA's relative to host mRNA is generally the case. T7 mRNA's have half-lives of up to 20 min (29, 42) and T4 mRNA, which previously had been considered unstable, has a half-life of 10 min, some five times greater than host mRNA (9).

Generation of φX mRNA's. Our results are in general agreement with those of Hayashi and Hayashi (20) and lead us to similar conclusions. (i) The largest size estimate for a φX-specific protein (15) is equivalent to an RNA mol wt of 0.5×10^6; therefore the majority of RNAs detected are polycistronic. (ii) Furthermore, when the sizes of peaks less than unit length are added, the value obtained is much greater than the unit length, implying that cistrons are represented in more than one peak. (iii) Molecules greater than the unit length would result from continued synthesis in the absence of termination or, more likely, from continuation of synthesis past a termination site (6).

It is possible that in vivo φX RNAs arise from endonucleolytic cleavage of larger precursor molecules as proposed for T7 mRNA (11). However, the efficiency of such a cleavage would have to be high since all RNAs were detected in both short and long pulses. The presence of molecules greater than unit length at longer pulse times argues against such an efficient cleavage mechanism. It is equally probable as Hayashi and Hayashi (20) suggested that these RNAs are the result of several discrete initiation and termination events. Such a model, coupled with the possibility of read-through of termination sites, provides the simplest system for variation of gene dosage in polycistronic messages.

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