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Process of Infection with Bacteriophage φX174

XXXV. Cistron VIII

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Twenty-two new amber and ochre mutants of φX174 were isolated and classified into complementation groups. Three ochre mutants gave positive complementation tests with reference mutants in the seven previously defined groups and thus represent an eighth cistron. Studies of the physiology of infection in the nonpermissive condition for mutants in cistron VIII yielded the following information. (i) Replicative-form synthesis proceeds at a normal rate, and is turned off at the usual time. (ii) Synthesis of single-stranded deoxyribonucleic acid (DNA) is delayed until nearly 40 min after infection (in the absence of lysis), at which time a slow synthesis of infectious phage particles commences. The synthesis of infectious particles at late times is interpreted as a consequence of "leakage," and indicates that the cistron VIII product is required in very small quantities. (iii) During the normal period of single-strand synthesis, most of the replicative-form DNA is found in a form with properties similar to those of the transient intermediates of single-strand DNA synthesized during normal infection.

Genetic analyses of previously isolated amber, opal, and temperature-sensitive mutants of φX174 indicated the presence of seven complementation groups (12). Seven complementation groups have also been found for the closely related bacteriophage S13 (15). There are about 1,800 codons in the deoxyribonucleic acid (DNA) of φX174 with the potential to code for about 2×10^5 daltons of protein. If it is assumed that the average molecular weight is 2×10^4 per gene product, up to 10 proteins might be coded by the φX174 DNA.

Ochre mutants of φX174 were isolated in an attempt to find new complementation groups. Ochre mutants were chosen because, as induced by C → T transitions, they are derived from a codon, CAA, different from those codons which give rise to amber or opal mutants. Three ochre mutants were isolated which complement reference mutants in all seven previously defined complementation groups but do not complement each other. These mutants thus define an eighth cistron. The physiology of infection in the nonpermissive condition was examined for these mutants and for other newly isolated ochre and amber mutants.

The preceding paper in this series (10) described the attachment and eclipse steps in the process whereby φX174 invades the host cell.

MATERIALS AND METHODS

Bacteria. *Escherichia coli* HF4704 (Su^-, Hcr^-; 4) and *E. coli* WWU (Su^-) and its derivatives which suppress amber phage mutants ($Su1_{am}$, $Su2_{am}$, and $Su3_{am}$) or amber and ochre phage mutants ($Su1_{oc}$, $Su2_{oc}$, and $Su3_{oc}$; 11) have been previously described. The several suppressor derivatives, Su1, Su2, and Su3, are believed to insert serine, glutamine, and tyrosine, respectively, at the site of the nonsense codon.

Media. KC broth, TPGA, and borate dilution fluid have been previously described (5).

Bacteriophage. Phage mutants used as reference stocks for complementation tests have been previously described (12) except for *am* N-1 and *op* 6. Mutant *am* N-1 was isolated by M. Hayashi, and *op* 6 was isolated by C. Hutchison (Ph.D. Thesis, California Institute of Technology, Pasadena, 1968).

Suppression of host DNA synthesis. Host DNA synthesis was suppressed with 50 μg of mitomycin C per ml (Nutritional Biochemicals Corp., Cleveland, Ohio) by the method of Lindqvist and Sinsheimer (9).

Induction of amber and ochre mutants. Amber and ochre mutants were induced from wild type φX174 by use of the mutagenic action associated with decay of ^{3}H-5-cytosine (4). Wild-type phage to be labeled with ^{3}H-5-cytosine were grown in TPGA medium in the presence of 2.5 μg of ^{3}H-5-cytosine per ml, 14.7 c/mmole (Schwarz BioResearch Inc., Orangeburg, N.Y.) in the host *E. coli* WWU. Radioactive progeny phage were diluted 100-fold into borate dilution fluid and stored at 5 C to accumulate decays. The stored phage were assayed for forward amber and ochre mutants over a period of time corresponding to one to two accumulated decays per phage.

Ochre mutants were also induced from the φX174 mutant *am3* by use of the mutagen hydroxylamine

(HA). Mutant *am*3, a lysis-defective mutant, was treated with HA according to the procedure of Tessman, Poddar, and Kumar (16), for a time sufficient to produce one lethal hit per phage. The use of *am*3 ochre double mutants is advantageous in that, in the nonpermissive condition, the effect of the ochre mutation can be studied late in infection without concern for lysis.

Forward ochre and amber mutants were screened by the double-layer technique described by Hutchison (Ph.D. Thesis, California Institute of Technology, Pasadena, 1968). The first layer of top agar contained the WWU Su^- host, and the second layer contained the test phage and the $Su2_{oc}$ suppressor. Wild-type parent phage will produce a plaque in both layers of agar, whereas the ochre or amber mutants will grow only on the top layer containing the ochre suppressor. Wild-type phage produce clear plaques, and the amber or ochre mutants show as cloudy plaques. Both layers were made with 1.5 ml of top agar. Isolation of *am*3, ochre double mutants from the *am*3 parent stock, was done in a similar manner, except that the first layer of top agar contained the $Su2_{am}$ suppressor rather than Su^- cells.

All turbid plaques were picked with a sterile toothpick and streaked on $Su2_{am}$, $Su2_{oc}$, and Su^- lawns. Stocks of phage from plaques which gave positive streak tests were made by inoculating a culture of the $Su2_{am}$ or $Su2_{oc}$ suppressor with a single plaque.

Complementation tests. The procedure used for the complementation tests was essentially as given by Hutchison and Sinsheimer (6). Phage stocks were diluted to 10^8 plaque-forming units (PFU)/ml in KC broth containing 3×10^{-3} M KCN. Samples of 0.4 ml of each of the two phage to be tested were mixed with 0.1 ml of *E. coli* HF4704 (at 10^8 cells/ml) which had been pretreated with 3×10^{-3} M KCN. The phage were adsorbed for 15 min at 37 C, and the culture was then chilled in an ice bath. Subsequently, 0.5 ml of ϕX174 antisera ($k = 100$ min^{-1}) was added; the mixture was incubated an additional 10 min at 37 C and returned to the ice bath. The infected cells were diluted 4×10^4-fold into chilled KC broth, incubated for 35 min at 37 C, chilled again, and plated for infective centers. The selfings were done in a similar fashion with the use of 0.8 ml of a single phage mutant per tube.

The time that the infected cells are held at growth temperatures must be carefully controlled, for longer incubation times may cause the occurrence of bursts of up to 50 *am*3 phage per cell. A similar difficulty with the single ochre mutants in cistron VIII was also encountered when longer incubation times were used. *E. coli* HF4704 was used as a host in these experiments because the ochre mutants in cistron VIII are, for unknown reasons, very leaky on the standard Su^- host *E. coli* C.

Thermal inactivation. Phage ϕX174 particles to be used in the heat inactivation tests were diluted into 2.5×10^{-2} M tris(hydroxymethyl)aminomethane (Tris) buffer (*p*H 8.1) to 5×10^7 PFU/ml. From these room temperature stocks, 0.2-ml samples were further diluted into 10 ml of Tris buffer preheated to the inactivating temperature. As a function of time, 1.0-ml samples were removed to empty tubes in an ice bath. The zero-time samples were diluted separately at room temperature.

Labeling and extraction of DNA. HF4704 was grown to 2.5×10^8 cells/ml in TPGA containing 2 μg of thymine per ml. A quantity of mitomycin C was added to provide 50 μg/ml. The cells were incubated without aeration for 30 min, and then were diluted to 5×10^7 cells/ml into fresh TPGA containing 1 μg of thymine per ml. To each of two 6-ml portions, 0.5 ml of ^{3}H-thymine (10.9 c/mmole, 0.5 mc/ml) was added. Each culture was infected with either *am*3 or *am*3,oc*12* at a multiplicity of infection (MOI) of 3.0. Time was measured from the time of infection. Samples (1 ml) were removed every 10 min to centrifuge tubes in an ice bath. The infected cells were pelleted in the cold in a Sorvall centrifuge, the supernatant fluid was removed, and the pellet was suspended in 0.50 ml of lysis mixture (10^{-1} M Tris, *p*H 8.0, 0.10 M NaCl, 0.2 mg of lysozyme per ml, 10^{-2} M ethylenediaminetetraacetate). After 30 min of incubation at ice-bath temperature, 10 μliters of Sarkosyl (30%, Geigy Chemical Corp., Ardsley, N.Y.), 50 μliters of Pronase (1 mg/ml), and 100 μliters of a mixture of purified replicative-form I, (RFI) and purified intact phage, both labeled with ^{14}C-thymine, were added. This mixture was incubated at 46 C for 12 hr and then was frozen until analyzed in an ultracentrifuge.

Centrifugation procedure. Preformed CsCl gradients of 5.0 ml each were prepared with a density range from 1.20 to 1.35 g/ml. A sample of 0.10 ml of each deproteinized lysate was applied to the top of each tube. The tubes were centrifuged in an L2-65B Spinco ultracentrifuge at 45,000 rev/min for 140 or 150 min with the brake off. Sufficient drops were collected per tube to make about 45 fractions.

All fractions were assayed for trichloroacetic acid-insoluble radioactivity in a Beckman LS-250 scintillation counter. The counter was adjusted to give about 13% ^{14}C overlap into the tritium channel. All data were adjusted to remove background and ^{14}C overlap in the tritium channel.

RESULTS

Derivation of ochre mutants. When decays of ^{3}H-5-cytosine (C $\rightarrow$ T transitions) were used as a mutagen, the relative frequency of induction of amber to ochre mutants was about 10. Presumably, the differential response reflects the relative frequency of CAG versus CAA glutamine codons in the ϕX174 messenger ribonucleic acid (RNA) giving rise, respectively, to amber (UAG) and ochre (UAA) codons. The approximate frequencies were 3×10^{-3} amber mutants per decay per phage and 3×10^{-4} ochre mutants per decay per phage.

Data from the complementation tests of the newly isolated amber and ochre mutants are given in Tables 1 and 2. The amber mutants are divided among cistrons II, VI, and VII. Ochre mutants occurred only in cistron IV and a new complementation group, cistron VIII.

TABLE 1. *Complementation tests of amber and ochre single mutants*[a]

Mutant gene	Selfing	Am3 I	AmN-1 II	Am9 III	Am14 IV	Am10 V	Am8 VI	Op6 VII	Oc6 VIII	Gene assignment
Am3		0.03								
AmN-1			0.02							
Am9				≤0.01						
Am14					≤0.01					
Am10						0.02				
Am8							0.2			
Op6								≤0.01		
Oc6									1.1	
Am80	≤0.01	14	*≤0.01*	18	≥50	≥50	≥50	27		II
Am90	≤0.01	≥50	*0.02*	4	≥50	30	30	20		II
Am82	0.10	38	≥50	42	≥50	10	*0.2*	≥50	24	VI
Am83	0.04	32	47	21	26	12	*0.2*	25	41	VI
Am84	0.03	29	19	21	26	10	*0.2*	30		VI
Am85	0.06	27	40	20	68	5.5	*0.1*	48		VI
Am86	0.05	20	30	35	31	19	*0.1*	19		VI
Am87	≤0.01	12	21	11	22	5	9	*≤0.01*		VII
Am88	≤0.01	16	19	7	18	4	8	*≤0.01*		VII
Am89	≤0.01	15	20	19	22	19	22	*≤0.01*		VII
Oc1	≤0.01	30	≥50	≥50	*≤0.01*	≥50	≥50	≥50	≥50	IV
Oc2	≤0.01	33	≥50	32	*≤0.01*	44	33	27		IV
Oc3	≤0.01	25	≥50	≥50	*≤0.01*	≥50	≥50	≥50		IV
Oc4	≤0.01	24	≥50	≥50	*≤0.01*	≥50	≥50	≥50		IV
Oc5	≤0.01	33	≥50	≥50	*≤0.01*	≥50	≥50	≥50		IV
Oc6	1.1	≥50	≥50	44	33	23	≥50	≥50		VIII
Oc7	1.8	≥50	≥50	≥50	≥50	28	≥50	≥50	*2.4*	VIII

[a] Complementation tests were done as described in Materials and Methods. The numbers given represent yields of phages per cell of both genotypes. Yields considered to indicate a lack of complementation are italicized.

TABLE 2. *Complementation tests of Am3,Ochre double mutants*[a]

Mutant gene	Selfing	Am3 I	Am80 II	Am9 III	Am14 IV	Am10 V	Am8 VI	Am87 VII	Oc6 VIII	Gene assignment
Am3		0.03								
Am80			≤0.01							
Am9				≤0.01						
Am14					≤0.01					
Am10						≤0.01				
Am8							0.15			
Am87								0.01		
Oc6									0.5	
Am3,Oc8	0.06	*0.10*	≥50	≥50	*≤0.01*	≥50	40	40	4	I, IV
Am3,Oc10	≤0.01	*0.10*	14	18	*≤0.01*	32	*0.4*?	12	12	I, IV, VI?
Am3,Oc12	≤0.01	*0.10*	≥50	≥50	≥50	≥50	40	41	*0.5*	I, VIII
Am3,Oc11	≤0.01	*0.01*	5	48	*≤0.01*	≥50	13	21	13	I, IV
Am3,Oc9	≤0.01	0.85?	12	31	*≤0.01*	39	4	5	6	I?, IV

[a] Complementation tests were done as described in Materials and Methods. The numbers given represent yields of phages per cell of both genotypes. Yields considered to indicate a lack of complementation are italicized.

Mutants in all cistrons except cistron VI complement in an essentially symmetric manner. That is, significant yields of both genotypes are always obtained. For complementation tests involving cistron VI mutants [as with cistron IV mutants of S13 (14)], the yields are asymmetric in that the cistron VI genotype is rescued very poorly, as shown by Sinsheimer, Hutchinson, and Lindqvist (13). The mutant *am*3,*oc*10 is unusual in the sense that, although it complements

mutants in cistron VI very poorly, complementation tests involving *am3,oc10* and mutants in cistrons other than VI produce symmetric yields of both genotypes.

Sinsheimer had previously reported (12) that mutants in cistron VII also complement in an asymmetric manner. This response appears to have been a consequence of a peculiarity of the mutant *ts*41D used in those tests, (*ts*41D appears to contain a second, weak *ts* mutation in cistron VI), since other mutants (*op6* and *am87*, *am88*, or *am89* in cistron VII) all complement in an essentially symmetric manner.

Properties of cistron VIII mutants. The selfings for the ochre mutants in cistron VIII were typically higher than those obtained for any other mutant tested, indicating a considerable degree of leakiness. The leakiness is more clearly indicated in Fig. 1, where the synthesis of total phage-specific DNA (detected with suppression of host DNA synthesis by mitomycin C) and progeny phage for a cistron VIII infection in the Su^- host HF4704 is shown. The *am3,oc12*-infected cells produced appreciable amounts of phage-specific DNA but only one phage per cell in 40 min of infection. Under these same conditions, the *am3* parent would produce about 100 intracellular phage in 40 min.

A comparison of the phage-specific DNA synthesized by the double mutants *am3,oc8* (cistron IV) and *am3,oc12* (cistron VIII), and by the parent *am3*, is presented in Fig. 2. The incorporation of ^{3}H-thymine into *am3,oc8*-infected HF4704 cells effectively ceased after about 20 min, as previously found for temperature-sensitive mutants in cistrons II, III, IV, and V (8, 9). However, *am3,oc12*-infected cells continued to make phage-specific DNA through 60 min of infection, although at a rate much lower than the *am3* control.

In an experiment similar to that shown in Fig. 2, the DNA was extracted from infected cells with Sarkosyl and Pronase at 10-min intervals up to 60 min after infection with either the *am3* or *am3,oc12* mutant. This material was sedimented on a preformed CsCl gradient. At 10 min after infection (data not shown), only RFI and RFII DNA was made in either infection. The sedimentation profiles were nearly identical. At 20 min after infection, no material sedimented in the single-stranded region of the gradient for the *am3,oc12* infection (Fig. 3). However, a

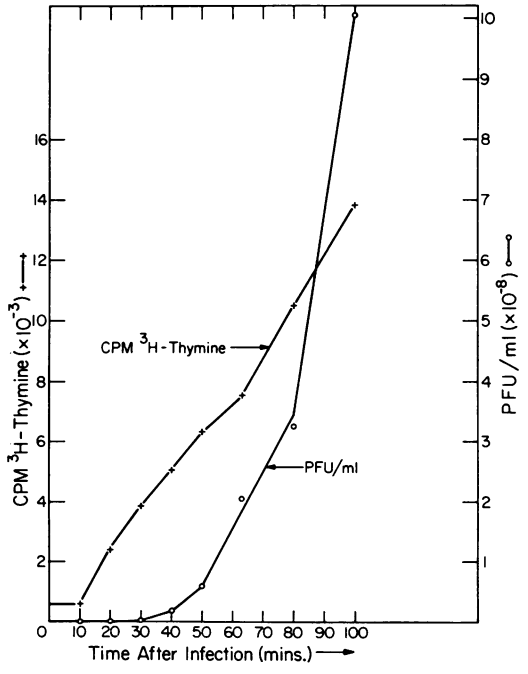

FIG. 1. *Synthesis of am3,oc12 phage-specific DNA and progeny phage in the nonpermissive conditions. HF4704 at 5 × 10⁷ cells/ml was infected with 5.0 am3,oc12 phage/cell. The cells were pretreated with 50 μg of mitomycin C per ml. ³H-thymine was added 1 min before infection to a specific activity of 2.7 c/mmole. Trichloroacetic acid-insoluble counts per minute of ³H-thymine, +; intracellular phage/ml, ○.*

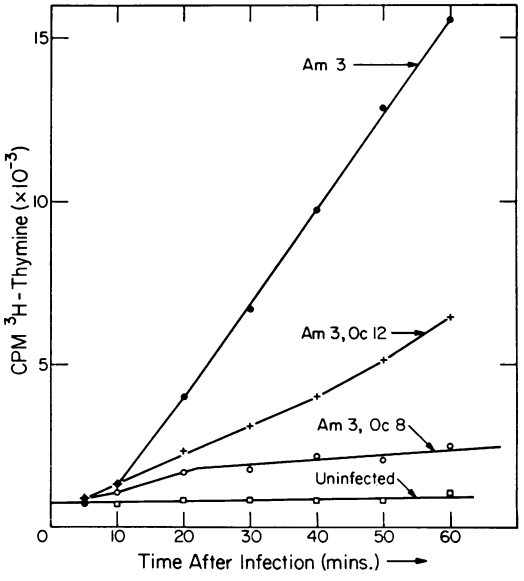

FIG. 2. *Synthesis of phage-specific DNA from am3,oc8 (cistron IV), am3,oc12 (cistron VIII), and am3 (cistron I) infected cells. Procedure same as Fig. 1 except that cells were 2 × 10⁷/ml and the specific activity of the ³H-thymine was 4.6 c/mmole. Trichloroacetic acid-insoluble counts per minute: am3, ●; am3, oc12, +; am3,oc8, ○; uninfected control, □.*

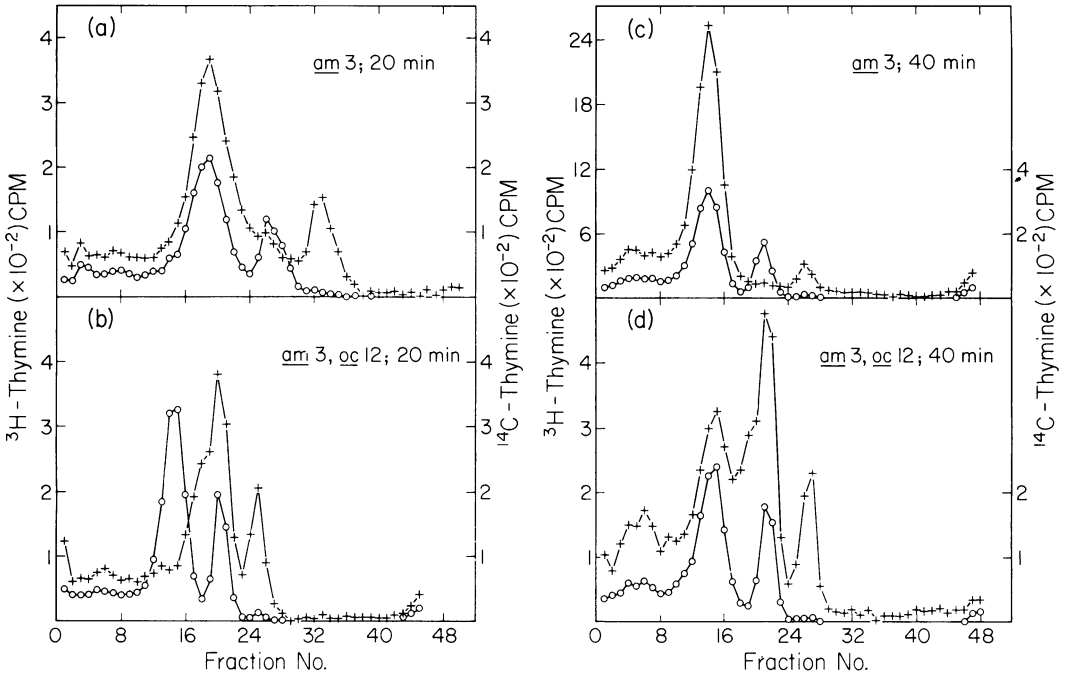

FIG. 3. *Sedimentation of phage DNA from HF4704 cells infected with am3 and am3,oc12 phage, and labeled with ^{3}H-thymine for 20 and 40 min. Infection, DNA extraction, and centrifugation as described in Materials and Methods. Trichloroacetic acid-insoluble counts per minute of ^{3}H-thymine, +; ^{14}C single-stranded and replicative-form DNA marker, ○. (a) am3, 20-min infection. (b) am3,oc12, 20-min infection. (c) am3, 40-min infection. (d) am3,oc12, 40-min infection.*

shoulder of material appeared on the leading edge of the RFI peak. This shoulder was present on every *am*3,*oc*12 gradient profile from 20 to 60 min after infection. A shoulder of material in front of the RFI marker was not obvious for the *am*3 profiles, although the large amount of single-stranded material present would make it difficult to detect. A small amount of single-stranded material appeared in the *am*3,*oc*12 gradient profile at 30 min after infection and steadily increased in amount thereafter.

There was a striking difference in the proportion of RF DNA which sedimented as RFI and RFII at 20 min and thereafter for the two infections. At 10 min, both mutants had synthesized predominantly RFI DNA. At 20 min and thereafter, the RF found in an *am*3 infection was principally RFII. In contrast, the RF component of an *am*3,*oc*12 infection sedimented predominantly as RFI and its leading shoulder, through the 60-min point.

The total counts per minute appearing in the RF regions of gradients from both infections are summed and plotted as a function of time after infection in Fig. 4. About 50% more material sedimented as RF molecules from the *am*3,*oc*12

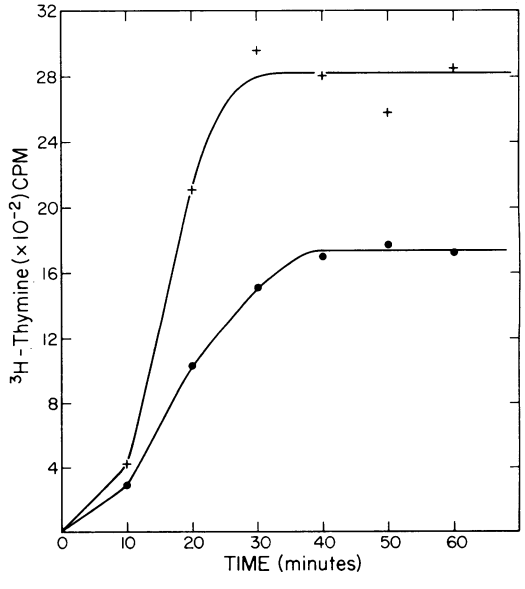

FIG. 4. *Total ^{3}H counts per minute incorporated into the replicative-form regions of gradients from the infected cultures of Fig. 3. Symbols: ●, am3; +, am3,oc12.*

infection as compared with the *am*3 infection for all time points after 10 min.

Measurement of the total incorporation of ^{3}H-thymine in HF4704 (Su$^-$) infected cells not treated with mitomycin C (data not shown) indicated that the *am*3,ochre mutants in cistrons IV and VIII effectively shut off host DNA synthesis at about 20 min after infection, as do all mutants tested to date (8).

The thermal sensitivity of the *am*3,*oc*12 mutant particles was assayed in an attempt to determine whether the cistron VIII protein might be present in the assembled phage. Since this mutant was induced by HA (C $\rightarrow$ T transitions), the codon from which the ochre codon was derived was almost certainly the glutamine codon, CAA. If the cistron VIII protein is present in the intact phage, *am*3,*oc*12 phage grown on the Su2$_{oc}$ suppressor inserting glutamine at the site of the ochre codon would behave as do *am*3 phage with regard to heat sensitivity. When grown on the serine or tyrosine ochre suppressors (Sul$_{oc}$ and Su3$_{oc}$, respectively), the mutant particles might be more thermally sensitive or more thermally resistant (1) than *am*3.

As shown in Fig. 5a, the thermal sensitivity of *am*3,*oc*12 tested at 59 C was the same when grown on any of the three ochre suppressors. Also, except for the initial greater rate of inactivation, the sensitivities were not significantly different from that for the wild-type control. A similar result was obtained for the single mutant *oc*6. For comparison, the thermal inactivation of *am*88 in cistron VII is shown in Fig. 5b. When grown on the ochre suppressor (Su3) inserting tyrosine, this mutant was much more sensitive to heat inactivation than when grown on the ochre suppressor (Sul) inserting serine. Mutant *am*88 was inactivated at 55 C rather than 59 C because of its greatly increased thermal sensitivity when grown on the Su3$_{oc}$ host. The mutants *op* 6 and *ts*41D in cistron VII had previously been shown to have a different thermal sensitivity than that of wild type ϕX174 (Hutchison, Ph.D. Thesis, California Institute of Technology, 1968).

DISCUSSION

When the mutagenic activity associated with the decay of incorporated ^{3}H-5-cytosine is used to generate nonsense mutants, the probability of producing a mutant per decay per phage is ca. 3×10^{-3} for amber mutants and ca. 3×10^{-4} for ochre mutants. From these mutation frequencies, an estimate can be made of the number of different triplets in the ϕX174 DNA which, after a C $\rightarrow$ T transition, will produce amber (UAG) or ochre (UAA) codons in the ϕX174 messenger

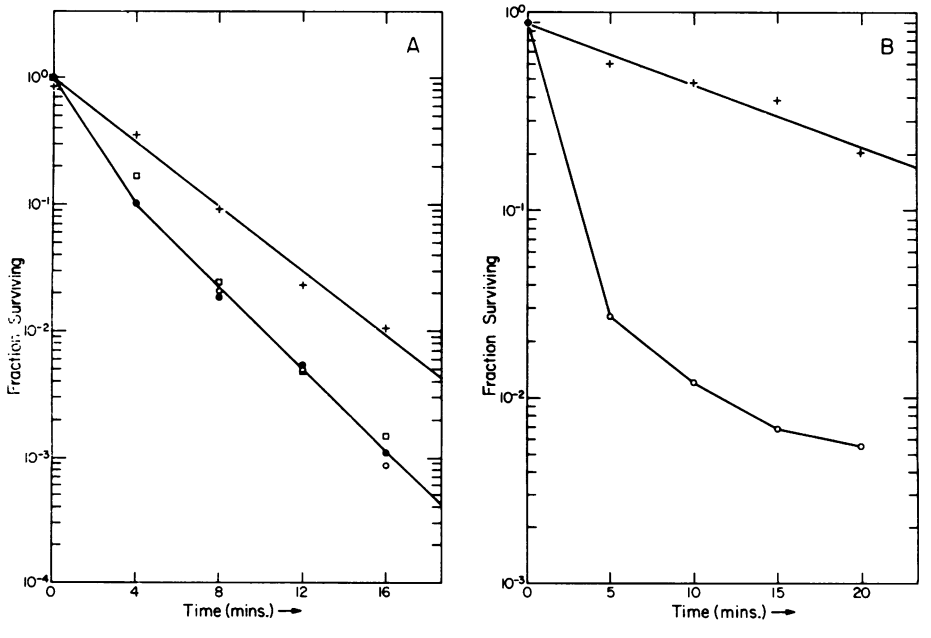

FIG. 5. *Heat inactivation of phage mutants grown on different suppressors, Procedure as given in Materials and Methods. (a) Inactivating temperature, 59.5 C. Wild type, +; am3,oc12 grown on Sul$_{oc}$, ○; Su2$_{oc}$, ●; Su3$_{oc}$, □, (b) Inactivating temperature, 55 C. Mutant am88 grown on Sul$_{oc}$, +; Su3$_{oc}$, ○.*

RNA. The frequencies of induction observed are a product of the probability of producing a mutant per site (at which an amber or ochre codon can arise by a C → T transition), times the number of sites per genome. The probability of producing a mutant by this decay (C → T transition) per single potential site in ϕX174 DNA is 2×10^{-4}. From the total probability for X number of sites, 3×10^{-3}, and the probability when $X = 1$, 2×10^{-4}, it appears that there are a total of 15 different sites at which amber mutants can occur by C → T transitions. For ochre mutants, the number of potential sites is $3 \times 10^{-4}/2 \times 10^{-4}$, or 1.5. This number must be two rather than one because separable mutants in cistrons IV and VIII already exist.

Implicit in making these calculations is the assumption that all those amber or ochre mutants which were induced were detected. Because of this assumption, the numbers of sites calculated here are probably minimal estimates.

Ochre mutants induced either by ^{3}H-5-cytosine decays or by treatment with HA fell into only two complementation groups: IV and a new group VIII. The ochre mutants in cistron VIII are unusual in the sense that they are very leaky on *E. coli* C, the normal Su$^-$ host used in this laboratory. The Su$^-$ hosts *E. coli* HF4704 and *E. coli* WWU are much more restrictive for the cistron VIII ochre mutants than is *E. coli* C. Even when grown on HF4704, however, the lysis-defective double mutant *am*3,*oc*12 can produce as many as 20 intracellular phage after 100 min. We interpret this leakiness to indicate that very little cistron VIII protein is required to obtain a successful infection.

HF4704 cells infected with the lysis-defective ochre mutant in cistron IV synthesize replicative-form molecules for about 20 min after infection, whereupon phage-specific DNA synthesis ceases. The turnoff of RF DNA synthesis by mutants of ϕX174 in the nonpermissive condition has been previously observed by Lindqvist and Sinsheimer (5, 6, 8). Infection with the double mutant of cistron VIII also exhibits this characteristic, at shown in Fig. 4. No mutant of ϕX174 yet tested fails to turn off replicative-form synthesis at about the time that single-stranded synthesis should be initiated.

There are several very interesting observations of the *am*3,*oc*12 infection, however. After 10 min of infection, a shoulder of material appears which sediments on the leading edge of the RFI peak. This is the area where replicative intermediates, RF molecules in the act of synthesizing single-stranded tails, have been shown to sediment (7).

The *am*3,*oc*8 mutant, like other mutants in coat protein cistrons which never synthesize single-stranded DNA in the nonpermissive condition, produces predominantly RFI DNA. The *am*3 control infection, which is actively producing single-stranded DNA, contains predominantly RFII replicative-form molecules, once single-stranded synthesis has been initiated. In contrast, the *am*3, *oc*12 infection contains an appreciable amount of material sedimenting in the single-stranded region by 40 min after infection, but the RF DNA continues to sediment predominantly as RFI plus the leading shoulder, even after 60 min.

The plot of total "RF-like" material made as a function of time (Fig. 4) indicates that the RF synthesis is shut off at approximately the same time for the *am*3,*oc*12 infection as in the *am*3 control, but 50% more material is found in the *am*3,*oc*12-infected cells.

We interpret these data to indicate that the absence of cistron VIII function results in a significant accumulation of RF molecules containing single-stranded tails. This conclusion is supported both by the observation that a shoulder of material is present which sediments faster than the RFI marker DNA and also by the fact that 50% more material sediments in the RF region of the gradient of the extract from the *am*3,*oc*12 infection than on that from the *am*3 infection.

We have not determined what fraction of the material sedimenting in the RFI region is actually RFI material and what fraction represents replicative intermediates attempting to make single-stranded DNA. In addition, we do not know whether the single-stranded material which accumulates in *am*3,*oc*12-infected cells after 30 min is made in all infected cells or only in some fraction of them. Finally, we do not know whether the escape from the nonpermissive condition is a consequence of a phage-directed product available in small quantities or in very low biological activity or to a host product which inefficiently substitutes for the phage products.

The extent of escape from the nonpermissive condition varies over a large range, dependent upon which Su$^-$ host is being utilized. Unfortunately, this is not helpful in determining the mechanism by which the infection escapes the nonpermissive condition. It is an indication, however, that the escape is probably not a result of accumulation of large quantities of protein fragments of low activity (ochre codon very near the end of the gene), since if this were the case no variation should be seen from one Su$^-$ cell to another.

The temperature-sensitive mutant *ts*4 in cistron II produces defective particles in the nonpermissive condition at 40 C which contain single-stranded DNA infectious to protoplasts (3). Cultures infected by the single mutants *am*80 (cistron II), *am*89 (cistron VII), and *oc*6 (cistron VIII) were examined, in the nonpermissive condi-

tion (HF4704 treated with 50 μg of mitomycin C per ml), to determine whether defective particles were produced. Up to 22 min after infection with *oc*6 or *am*89, no material was produced containing ^{3}H-thymine which sedimented faster than RFI in a sucrose gradient. Mutant *am*80 infections, however, produced material containing ^{3}H-thymine which sedimented in a sharp band at about 108*S* and in a broader band at about 80*S* (data not shown). The bulk of the ^{3}H-thymine counts were in the 80*S* band. These results are very similar to those obtained by Krane for the *ts*4 mutant in experiments in which an immunological assay for phage coat antigen was used (S. Krane, Ph.D. Thesis, California Institute of Technology, Pasadena, 1966).

The thermal sensitivity of the cistron VIII mutant, *am*3,*oc*12, was studied in an attempt to ascertain the presence of the cistron VIII protein in the phage particle. Interpretations of such studies can be confounded by the possibility that there may be second, sublethal mutations in the phage which are responsible for any altered sensitivity observed. Differential comparison of the thermal stabilities of phage stocks grown on a variety of ochre suppressors which insert different amino acids provides an internal control which circumvents this difficulty. With the cistron VII amber mutant, such studies provided further evidence that this cistron specifies a coat protein; with the ochre mutant in cistron VIII, the thermal sensitivities of the phage produced were identical when the mutant was grown on hosts with three different ochre suppressors. The latter result suggests that the cistron VIII protein is not present in the assembled phage, but does not, of course, prove this to be the case.

Studies of the phage-specific proteins made in *am*3,*oc*12 infections under nonpermissive conditions have been analyzed by acrylamide gel electrophoresis. The protein patterns obtained were not significantly different from those obtained for an *am*3 infection, again suggesting that the undetected cistron VIII product may be present in very small quantities (R. Mayol, *personal communication*).

Tessman et al. (15) have reported an ochre mutant representing a seventh cistron (VI) in the closely related phage S13, but no data are available concerning its DNA synthesis. Cistron VI of S13 and this cistron VIII of φX174 could possibly direct identical functions, even though the late "leakiness" characteristic of the cistron VIII ochre mutants was not observed with the S13 mutants.

It seems most likely that the function of the cistron VIII protein is connected with the synthesis of single-stranded DNA, possibly at a late stage. Several functions necessary for single-stranded DNA synthesis have been postulated (7) for which the cistron VIII function could be a candidate.

Burgess and Denhardt (2) estimated that the total protein molecular weight (from electrophoretic mobilities of defined protein in acrylamide gels) coded by φX174 cistrons I through VII is about 1.5×10^5. Assuming an average molecular weight of 110 per amino acid, the 1,800 codons in φX174 DNA could possibly code for a total protein molecular weight of 2×10^5. Hence, there could be as many as two or three cistrons not yet identified.

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