JOURNAL OF VIROLOGY, Mar. 1970, p. 282-288
Vol. 5, No. 3

Process of Infection with Bacteriophage φX174

XXXIII. Templates for the Synthesis of Single-Stranded Deoxyribonucleic Acid

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Received for publication 6 October 1969

The origin of the templates for the synthesis of φX174 progeny single-stranded deoxyribonucleic acid was studied by means of the mutagenic activity associated with the decay of incorporated ^{3}H-labeled 5-cytosine. The results indicate that the single-strand synthesis occurs in an asymmetric semiconservative manner using as template the complementary strands of the pool of replicative form molecules accumulated during the eclipse period. These complementary strands are repeatedly used as templates, and there is no detectable preferential use of complementary strand templates made early in the eclipse versus those made late.

During the course of bacteriophage φX174 infection, the parental single-stranded deoxyribonucleic acid (DNA) penetrates the bacterial cell and is converted to a double-stranded replicative form molecule (designated parental RF) (15). In starved cells or in cells grown in minimal medium, the parental RF molecules become attached to a limited number of replication sites (6, 18; M. J. Yarus, Ph.D. Thesis, California Institute of Technology, 1966), most often one per cell, and replicate in a semiconservative fashion producing a pool of progeny RF molecules which do not themselves replicate (2, 6, 16). The parental viral strand remains bound to the replication site, and, since only one RF per cell is undergoing synthesis, the pool of RF molecules increases in a linear fashion (2).

At the end of the eclipse period, the synthesis of progeny RF ceases, host DNA synthesis ceases, and synthesis of progeny single-stranded DNA and mature phage particles begins (8, 9). According to Komano, Knippers, and Sinsheimer (5, 7), the synthesis of progeny single-stranded DNA is an asymmetric semiconservative process in which the complementary strand of the progeny RF molecules is repeatedly used as a template.

The experiments reported here were designed to verify this mode of single-strand replication by a genetic analysis and to study some of the properties of the complementary strand template. The procedure involves the use of the mutagenic action associated with the decay of incorporated ^{3}H-5-cytosine. This mutagen has the capability, unique among all other mutagens, of mutagenizing only that nucleic acid containing the tritiated precursor (13). In addition, it causes only cytidine to thymidine (C → T) transitions (10; F. Funk and S. Person, *unpublished data*). By proper selection of mutants, the result of mutation in the viral or the complementary strands can be studied separately. Temperature-sensitive phage mutants which revert to wild type by C → T transitions in the viral strand can be used to study the effects of mutagenizing the viral strand of the RF molecules; mutants which revert to wild type by guanine to adenine (G → A) transitions in the viral strand (hence C → T transitions in the complementary strand) can be used to study the effects of mutagenizing the complementary strand.

In the model of φX174 single-strand replication proposed by Knippers, Komano, and Sinsheimer, the complementary strands of the progeny RF molecules which are used as the templates to produce the progeny single strands (5, 7) are made almost entirely during the eclipse period. There is almost no synthesis of new complementary strands after single-strand synthesis has been initiated. ^{3}H-5-cytosine incorporated into φX174-infected cells during the eclipse period labels both viral and the complementary strands of the progeny RF. For a mutant reverting by a G → A transition, such labeling of the complementary strand of the progeny RF molecules should lead (after ^{3}H decay) to the production of revertant phage. However, incorporation after the eclipse period should be ineffective as only plus strands are being synthesized during this period. In addition, if the complementary strands of the progeny RF molecules made during the eclipse period are the only templates used for progeny single-strand synthesis, and if these are uniformly labeled during the eclipse period, the label is then removed,

and the cells immediately stored to allow decay before incubation, then one would predict that the reversion frequency (the fraction of the progeny phage which are revertant) would remain constant if cultures are sampled throughout the subsequent posteclipse period.

Infected cells were pulsed with ^{3}H-5-cytosine at various times during the latent period. They were subsequently filtered to remove unincorporated tritium, frozen, and stored at −65 C to accumulate decays. Samples were thawed as a function of time, reincubated to complete the infection, and lysed with lysozyme and ethylenediaminetetraacetic acid (EDTA), and the progeny phage was assayed at 30 and 41 C.

The previous paper in this series is Newbold and Sinsheimer, J. Mol. Biol., 1970, *in press*.

MATERIALS AND METHODS

Bacterial stocks. *Escherichia coli* WWU is a strain 15 multiauxotroph requiring thymine, cytosine (or uracil), tryptophan, proline, methionine, and arginine (13). *E. coli* WWU Su 1_{am}, a derivative of WWU, contains an amber suppressor which inserts serine at the site of the amber codon (11). *E. coli* HF4714, the gift of C. Hutchison, carries an unknown amber suppressor effective in suppressing φX174 *am*3 mutants and was used as a plating indicator in these experiments. *E. coli* HF4704A requires thymine and cytosine (or uracil). It was derived from HF4704 (8) by treatment with nitrosoguanidine.

Phage stocks. Phages φX *am*3, *am*3*ts*γ, *am*3*ts*41D, and *am*3*ts*4 which have been previously described (14) were the gift of C. Hutchison. Phage *am*3*ts*163 was isolated from an *am*3 parent after treatment with nitrous acid.

Media. Starvation buffer (SB; 2), KC broth, top agar, and bottom agar (3) have been previously described. TPGA is the TPG previously described (16) less the $FeCl_3 \cdot 6H_2O$ plus 3.0 g of Vitamin Free Casamino Acids (Difco) per liter. Borate dilution fluid is 0.05 M sodium tetraborate. Lysis mixture is 0.2 mg/ml of lysozyme, 10^{-2} M disodium ethylenediaminetetraacetate in borate dilution fluid.

Labeling procedure. *E. coli* WWU or *E. coli* 4704A were grown to 2×10^8/ml in TPGA plus the necessary supplements. The infection was synchronized either by use of 3 mM KCN or by suspending the cells in SB for 60 to 90 min prior to infection by the method of Denhardt and Sinsheimer (1). The multiplicity of infection was 5. Medium containing ^{3}H-5-cytosine was added to infected cells either after filtration on 0.45 μm membrane filters (Millipore Corp., Bedford, Mass.) or by adding an equal volume of the radioactive medium to the infected cells. In all cases, the concentration of cytosine was 2.5 to 3.0 μg/ml at specific activities of 5.5 to 17 c/mmole.

After labeling, the cells were placed in an ice bath, filtered, and washed on membrane filters at 0 C and resuspended in chilled unlabeled medium. Samples of 0.2 ml were frozen in a dry ice-acetone bath and stored at −65 C in screw-cap vials to accumulate decays. Samples thawed as a function of time were incubated without aeration for 150 to 180 min at 30 C and then lysed by addition of lysozyme-EDTA [0.2 mg/ml of lysozyme, 10^{-2} M EDTA (*p*H 8.0)].

Incorporation of bromodeoxyuridine (BUdR). *E. coli* WWU Su I_{am} cells at 10^7/ml were infected with mutant phage at a multiplicity of infection of 10^{-3}. Infected cells were grown in KC broth containing 40 μg/ml of BUdR and 20 μg/ml of thymidine for 2.5 hr.

RESULTS

Classification of φX mutants. Nineteen double mutants containing the *am*3 (lysis-defective) mutation and a temperature-sensitive mutation were classified according to the base transition required for reversion of the *ts* character. The classification, given in Table 1, was determined by the response of the 19 mutants to the chemical mutagens hydroxylamine (HA), ethylmethane sulfonate (EMS), and bromodeoxyuridine (BUdR) (4, 19). Mutants in group I are assumed to respond to C → T transitions in the viral strand, group II by G → A transitions, and group III by A → G or T → C transitions. *Am*3*ts*γ and *am*3*ts*41D were chosen as representative of the class reverting (for temperature sensitivity) by C → T transitions. *Am*3*ts*4 and *am*3*ts*163 were chosen as representative of the class reverting by G → A transitions.

The loss of plaque-forming ability of *am*3*ts*γ as a function of accumulated disintegrations per phage is shown in Fig. 1. The inactivation follows single-hit kinetics as observed earlier for the related phage S13 (F. Funk and S. Person, *unpublished data*). The killing efficiency, α, the probability of inactivating a phage per decay, is about 0.5 for phage stored at 5 or −60 C. The rate of accumulation of decays per phage was calculated from the specific activity of the medium and the cytosine content of φX174.

The mutational response of intact *am*3*ts*γ and *am*3*ts*4 to decays of ^{3}H-5-cytosine is shown in Fig. 2. The fraction of the surviving phage which are revertant is plotted against the number of accumulated disintegrations per phage. The slope of the curve or the reversion frequency is the probability of producing a revertant per decay per phage. The mutant *am*3*ts*γ (C → T) responds quite strongly to decays of ^{3}H-5-cytosine, giving a reversion frequency of 2×10^{-4}; for *am*3*ts*4 (G → A), the reversion frequency is about 5×10^{-7}. Analogous results were obtained by using *am*3*ts*41D and *am*3*ts*163, as expected from the chemical mutagen characterizations.

Intracellular labeling. The results of labeling (with ^{3}H-5-cytosine) cells infected with *am*3*ts*4 during the period of progeny RF synthesis (10 to 20 min) and after RF synthesis has ceased (40 to 50 min) are given in Fig. 3. The end of the eclipse

TABLE 1. *Response of am3 temperature-sensitive mutants to treatment with HA, EMS, and BUdR: reversion frequencies of φX mutants at D_{37} ($\times 10^8$)*[a]

Group	Mutant	Spontaneous	HA	EMS	BUdR	Base change inferred
I	*am3tsγ*	300	*60,000*	—[b]	—	
	*am3ts*41D	200	*66,000*	—	—	
	*am3ts*142	200	*40,000*	—	—	
	*am3ts*146	400	*35,000*	—	—	C → T
	*am3ts*147	1,000	*50,000*	—	—	
	*am3ts*149	200	*40,000*	—	—	
	*am3ts*157	1,400	*80,000*	—	—	
II	*am3ts*4	20	≤20	*1,300*	≤20	
	*am3ts*162	200	≤100	*1,000*	—	
	*am3ts*163	300	≤150	*1,000*	—	G → A
	*am3ts*152	60	≤50	*1,400*	—	
	*am3ts*156	150	≤75	*1,100*	—	
III	*am3ts*379	30	≤30	≤30	*800*	A → G or
	*am3ts*9	20	≤30	≤30	*1,200*	T → C
	*am3ts*154	30	≤30	≤80	≤30	
	*am3ts*160	300	≤150	≤150		
IV	*am3ts*142	100	*1,100*	—	—	
	*am3ts*159	400	*760*	—	—	?[c]
	*am3ts*166	12	*900*	≤60	—	

[a] Mutation frequency is given as the net number of revertants occurring per 10^8 surviving phage after subtraction of the spontaneous frequency. In the case of treatment with HA or EMS, the mutation frequency reflects the induction at 37% survival. In those cases where significant induction occurred, the mutation frequencies are in italics.

[b] Not measured.

[c] These patterns of induced mutation cannot be explained at this time.

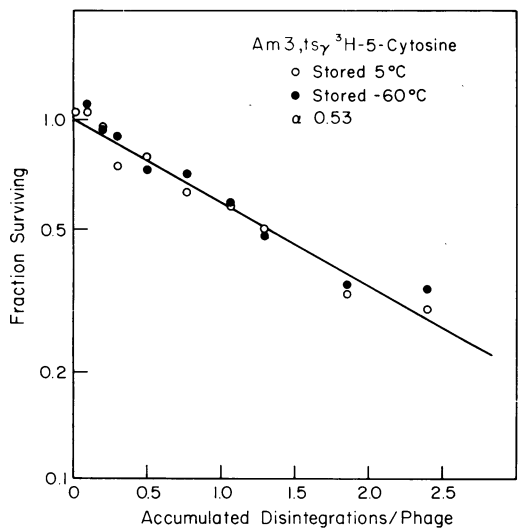

FIG. 1. *Loss of plaque-forming ability of am3tsγ from decays of ³H-5-cytosine.*

period, defined as the time at which an average of one intracellular phage per cell has accumulated, occurred at 25 min in this experiment. All times are counted from the time of removal of the synchronization conditions, i.e., KCN or SB. Cells infected with *am3ts*4 (G → A) respond to decays of ³H-5-cytosine incorporated during the period of active progeny RF synthesis (10 to 20 min), but do not respond (to ³H-5-cytosine incorporation) after the eclipse period (40 to 50 min). Data for cells labeled with ³H-6-uracil are shown here as a control indicating that the positive mutagenic response is due to the specific effect of ³H-5-cytosine decay. The decays of ³H in ³H-6-uracil, presumably incorporated into the phage DNA as ³H-6-cytosine (F. Funk and S. Person, *in press*), are less than 1% as effective in causing C → T transitions as are decays of ³H in ³H-5-cytosine.

The mutagenic response to decays of ³H-5-cytosine incorporated in several 5-min pulses during the eclipse period is shown in Fig. 4. The reversion frequency is plotted versus the time of the pulse of ³H-5-cytosine. The reversion frequency is constant for pulses at 0 to 5, 5 to 10, and 10 to 15 min. It then declines significantly for labeled cytosine incorporated during the pulses of 15 to 20, 20 to 25, and 25 to 30 min. The end of the eclipse period occurred at about 17 min in this experiment.

Because there is always a background of residual parental virus, when cells are labeled during

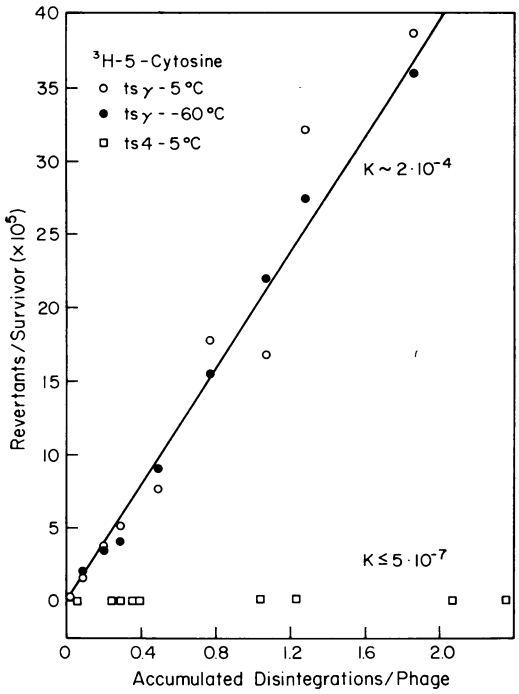

FIG. 2. *Reversion of the temperature-sensitive locus of am3tsγ and am3ts4 from decays of ^{3}H-5-cytosine. The slope of the curves or k value represents the probability of producing a revertant per decay per phage.*

the eclipse period, the observed fraction of resultant revertant phage is at first seen to increase and then remain constant as a function of the time of poststorage incubation subsequent to the eclipse period. In the experiment shown in Fig. 5, an infected culture was labeled with ^{3}H-5-cytosine from 10 to 20 min after infection and stored at −60 C for a time sufficient to accumulate one decay per labeled complementary strand. It was then thawed, incubated, and sampled at various times. The reversion frequency at zero time (no poststorage incubation) is very low and rises to nearly its maximal value at 30 min, after which it remained constant. Phage synthesis continued in this experiment until about 150 min.

Stability of decayed cytosine. An experiment was designed to determine the stability of those decayed cytosine bases which are transcribed as thymine in the process of replication of the viral single strand. The infecting viral strand remains bound to the hypothetical replication site and presumably is copied repeatedly in synthesis of the pool of progeny RF molecules. If a decayed cytosine base is always copied as thymine, then cells infected with *am3tsγ* or *am3ts*41D phage which have undergone a decay (in that cytosine for which reversion can occur) should synthesize a pool of only revertant RF molecules and should produce only revertant progeny phage. On the other hand, if the decayed base were read sometimes as thymine and sometimes as cytosine, cells infected with phage containing a decayed cytosine at the revertant site should contain a mixed population of mutant and revertant RF molecules and should give rise to a mixed burst of mutant and revertant phage. The experiment shown in Table 2 was designed to distinguish between these two possibilities.

A preparation of *am3tsγ* phage which had a reversion frequency of about 5×10^{-4} as a consequence of ^{3}H-5-cytosine decay was used to infect *E. coli* HF4704A in KCN at a multiplicity of infection of phage particles of about 0.10. The KCN was removed by filtration, and the cells

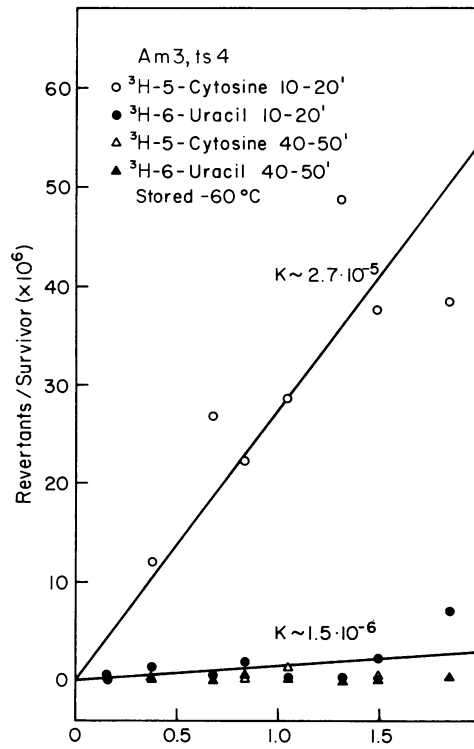

FIG. 3. *Reversion of the temperature-sensitive locus of am3ts4 as a consequence of tritium decays, accumulated in the infected cell. Infected cells were incubated in the presence of ^{3}H-5-cytosine or ^{3}H-6-uracil from 10 to 20 min or from 40 to 50 min after infection. Immediately after the labeling period, the samples were frozen and stored at −60 C to accumulate decays. Samples were thawed as a function of time and reincubated 180 min to complete the phage synthesis. Symbols: ○, ^{3}H-5-cytosine, 10 to 20 min; ●, ^{3}H-6-uracil, 10 to 20 min; △, ^{3}H-5-cytosine, 40 to 50 min; ▲, ^{3}H-6-uracil, 40 to 50 min.*

were resuspended and diluted in KC broth. The diluted culture was dispensed in 0.5-ml samples to give about 400 infected centers per tube. The tubes were incubated for 90 min at the nonpermissive temperature, 40.5 C, and lysed with lysozyme and EDTA. Half of each tube was plated on each of two plates, one subsequently incubated at 30 C, the other at 41 C. The number of plaques appearing on these plates for those tubes which gave revertant bursts is shown in Table 2. If the decayed cytosine base is always copied as thymine, then all of the progeny of such phage should be revertant and the number of plaques on the plates incubated at 30 C should be equal to the number on the plates at 40 C plus the average number which appear on the 30 C plates for a tube containing no revertant phage (counted), i.e., 32. (In addition the numbers in the 30 C column should be slightly greater than those in the 40 C column, plus 32, because of the slightly

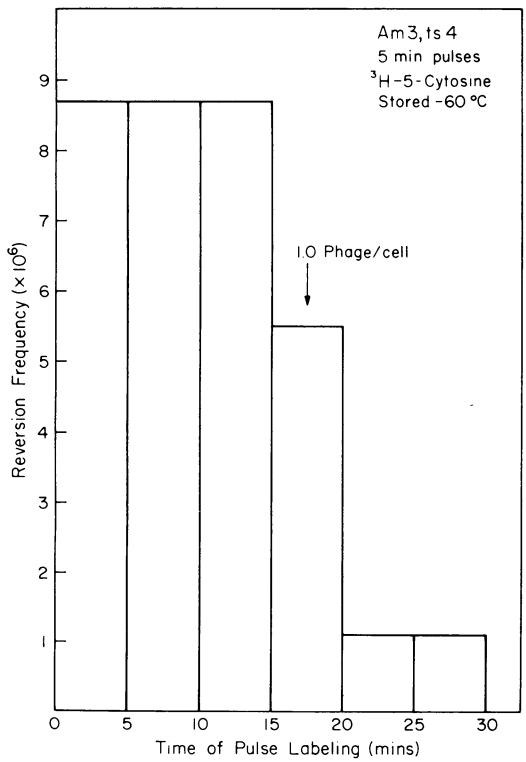

FIG. 4. *Reversion frequency of am3ts4 as a function of the time of pulse labeling with ^{3}H-5-cytosine. Cells infected with am3ts4 were pulsed with ^{3}H-5-cytosine for 5 min at 0, 5, 10, 15, 20, and 25 min after infection, immediately frozen, stored at −65 C, thawed, and reincubated, and the reverted frequency was measured in the final phage yield. An average of one phage per cell had been synthesized at 18 min.*

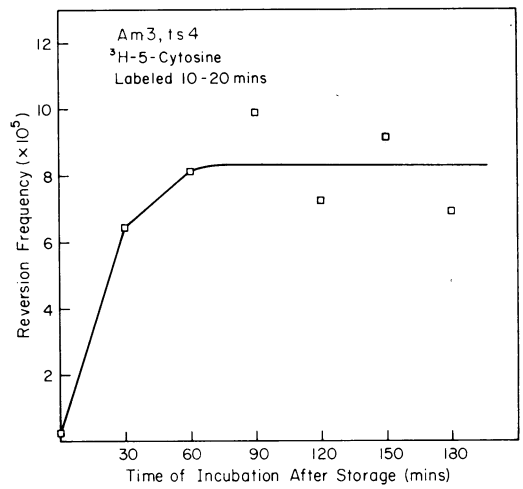

FIG. 5. *Reversion frequency as a function of the length of poststorage incubation. The labeling procedure was the same as in Fig. 3. The reversion frequency was measured as a function of the time of poststorage incubation for a constant number of accumulated decays.*

TABLE 2. *Single-burst analysis am3tsγ (^{3}H-5-cytosine), 48 total tubes, 400 infective centers per tube*[a]

Revertant bursts	
30 C	41 C
137[b]	121[c]
158	157
193	179
221	211
143	151
140	135
151	143
28	4
34	4
17	12

[a] Modified single-burst analysis of cells infected at the nonpermissive temperature with *am3tsγ*, having an average accumulation of 2.5 ^{3}H-5-cytosine decays per phage. The data under revertant bursts indicates the plaques on the pairs of plates incubated at 30 and 41 C for those pairs in which the 41 C plate contained plaques. Those pairs containing no plaques on the 41 C plate had an average of 32 plaques on the 30 C plate as a consequence of the escape synthesis by mutant phage. The following data are from the control in which unlabeled phage were used: average plaques per plate, 30 C; mean nonrevertant burst, 32 at 30 C and 0 at 41 C

[b] Mutants and revertants.

[c] Revertants only.

higher plating efficiency of φX174 *am*3 at 40 C over that at 30 C.) Results similar to those shown for *am3tsγ* were also obtained for *am3ts*41D. In all, over 60 bursts of revertant phage were examined with results similar to those shown here.

DISCUSSION

The mutants in Table 1 were classified according to the findings of Tessman, Poddar, and Kumar (17) and Howard and Tessman (4) for the closely related phage S13. Mutants which respond to HA are assumed to revert by C → T transitions. Mutants which respond to EMS, but not HA, probably revert by G → A transitions. Tessman found that EMS can cause T → C transitions at somewhat reduced efficiency. However, the response of those mutants classified in Table 1 as reverting by G → A transitions is fairly uniform; none revert at a greatly reduced efficiency. Mutants reverting by T → C transitions should respond to incorporation of BUdR, whereas G → A mutants may not (4). The mutant *ts*4 does not respond to BUdR, whereas two of three mutants classified as reverting by A → G or T → C do respond to BUdR. The mutants *tsγ*, *ts*41D, *ts*163, and *ts*4 were chosen for further tests with ^{3}H-5-cytosine to verify their classifications.

Tritium decay occurring in the DNA of S13 causes inactivation of plaque-forming ability with an efficiency of about 0.5 (F. Funk and S. Person, *unpublished data*). As for S13, a single event inactivates φX174, and, as for S13, the efficiency of inactivation per decay is about 0.5. The killing efficiency is the same when the phage are stored at 5 C or at −60 C.

As predicted from Table 1, the data in Fig. 2 indicate that *am3tsγ* and *am3ts*41D (data not shown) respond to decays of ^{3}H-5-cytosine, whereas the mutant *am3ts*4 does not. The reversion frequency for *am3tsγ* stored at 5 C or −60 C is 2×10^{-4}, while that for *am3ts*4 is about 5×10^{-7}. The reversion frequency for *am3tsγ* from ^{3}H-5-cytosine decay is not as high as that previously observed (8×10^{-4}) for the S13 mutant H_i1 (F. Funk and S. Person, *unpublished data*). It is not clear at this time why the response to ^{3}H-5-cytosine of φX174 is less than that of S13. Both the responses to HA and to EMS were uniformly higher for φX174 than had been reported for S13 (17).

In contrast to the result obtained with the viral DNA, the mutant *am3ts*4 does respond to the decay of ^{3}H-5-cytosine in the RF molecule (Fig. 3). By inference, the effective decay must be in the complementary strand. From this result, we confirm that the progeny single-stranded DNA is synthesized using the complementary strand of the progeny replicative form as a template as previously described by Knippers, Komano, and Sinsheimer (5, 7). Specifically, this conclusion is based on the following results. The *am3ts*4 mutant (G → A) does not respond to decays of ^{3}H-5-cytosine in the viral strand, whereas a mutant expected to respond (*am3tsγ*) did so (Fig. 2); the *am3ts*4 mutant did respond when the ^{3}H-5-cytosine was incorporated during the period of active RF synthesis (eclipse period, 10 to 20 min), but did not respond when the label was incorporated after the RF synthesis had ceased (40 to 50 min).

We also conclude that the complementary strand template is repeatedly used, since the final reversion frequency is quickly achieved and maintained at a constant value throughout the poststorage incubation (Fig. 5). If the templates were not repeatedly used, new templates would have to be continually synthesized to replace those which were discarded. Such new templates, made during the poststorage incubation, would not give rise to revertant phage and thus we would expect the curve in Fig. 5 to attain a maximum value early in the incubation and then decline.

In some experiments analogous to that shown in Fig. 5, a decline late in infection was in fact observed. This may be a consequence of RF turnover or it may be a consequence of the gradual diluting effect of newly made RF molecules [which continue to be made, but at a greatly reduced rate ($\leq 5\%$), after the end of the eclipse period]. In either case, we can conclude that the complementary strand template is not turned over with an appreciable frequency in up to 90 min of synthesis at 30 C.

The probability of use of a complementary strand as a template for progeny single-strand synthesis appears to be independent of the time at which the complementary strand was made (Fig. 4). The reversion frequency per 5-min pulse of ^{3}H-5-cytosine is constant through the first three pulses, reflecting the constant probability that any complementary strand will be used as template. The decline in reversion frequency for the remaining three pulses reflects decreased incorporation into RF accompanying the shutoff of progeny RF synthesis at the end of the eclipse period.

The chemical product arising in DNA from decay of ^{3}H-5-cytosine is stably recognized as thymine (Table 2). The stability is such that it will be copied as thymine at least 90% of the time. The background of mutant phage which escape at the nonpermissive temperature prevents a more accurate determination.

ACKNOWLEDGMENT

This research was supported in part by Public Health Service grant GM13554 from the U.S. Public Health Service.

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