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ϕX 174 Replicative Form DNA Replication, Origin and Direction

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Heteroduplex replicative form of bacteriophage ϕX174 was prepared by annealing minus strand isolated from wild-type ϕX RF† with plus strand DNA, obtained from temperature-sensitive and amber mutants of ϕX in different cistrons. Three different kinds of bursts were produced by spheroplasts infected with heteroduplex RF: bursts with only wild-type phage, bursts with only mutant phage and bursts with wild-type and mutant phage.

The frequencies of these bursts for RF with heteroduplex regions in different cistrons were determined in single-burst experiments under permissive conditions. A gradient in the percentage of spheroplasts producing both genotypes, depending on the position of the mutation in the plus strand of heteroduplex RF on the genetic map of ϕX phage, was observed. The direction of replication of double-stranded ϕX DNA as well as the origin of replication were deduced from this gradient.

1. Introduction

A number of different biochemical and genetic methods have been used to determine the direction and the origin of DNA replication in bacteria and phages. The rationale behind most of the methods is a "gene-dosage effect". Genes near the origin are present in a duplicated form during a greater part of the replication cycle than genes near the terminus. The direction of replication can be determined by measuring the time of appearance of duplicated genes with known positions on the genetic map during the replication cycle. The replication of a particular gene can be measured either directly by hybridization or transduction or indirectly from the amount of gene product, for example, enzyme (Caro & Berg, 1969; Masters, 1970; Makover, 1968; Tomizawa & Ogawa, 1968).

Another approach involves direct labelling of the origin or the terminus by density or radioactive labels, or by inflicting mutations specifically at the replication point after synchronization of DNA replication (Abe & Tomizawa, 1967; Wolf, Pato, Ward & Glaser, 1968; Cerdá-Olmedo & Hanawalt, 1968).

Recently, Schnöss & Inman (1970) analysed replicating branched circles of λ DNA by electron microscopy and determined the positions of the branched points relative to specific regions of the λ molecule which denature easily. The origin of DNA replication could be localized with high precision and the majority of the molecules replicated bidirectionally.

† Abbreviation used: RF, replicative form.

Mosig (1970) deduced the origin and replication direction of T4 DNA from a comparison of the recombination frequency of complete and incomplete T4 chromosomes. Stevens, Adhya & Szybalski (1971) determined the origin of autonomous λ DNA replication by hybridization of RNA from specific regions of the λ genome with DNA isolated after induction of lysogens, containing different deleted prophages.

This paper describes a new method for the determination of the direction and the origin of DNA replication. The method is based on the finding that the majority of heteroduplex φX RF, in which the plus- and minus-strand each contains a different genetic marker after addition to spheroplasts, is converted by a repair process to homoduplex RF before replication. As a consequence, these spheroplasts produce a single type of phage either of the plus-strand or of the minus-strand genotype. In a fraction of spheroplasts, heteroduplex RF escapes the repair process by replication and these spheroplasts produce phage of both genotypes (Baas & Jansz, 1971,1972). It is shown that as a result of competition between repair and replication, the percentage of spheroplasts producing both genotypes depends on the position of the heteroduplex region on the genetic map of bacteriophage φX174. Using this method, we have determined the direction and the origin of the DNA replication of double-stranded DNA of bacteriophage φX174. This method can be used in the determination of the direction and the origin of DNA replication of other DNA phages. An application of this method requires a biological assay of the DNA and a separation of the complementary strands.†

2. Materials and Methods

(a) E. coli *strains*

Strain C the usual host of φX was obtained from Dr R. L. Sinsheimer.
CR34-C416 (*su*$^+$) was used as a host for amber mutants of φX.
K58 (*su*$^+$) is a φX-resistant strain used for the spheroplast assay for φX DNA.

(b) *φX strains*

ϕ_0 is the wild-type φX;
φX *ts4* is a temperature-sensitive mutant in cistron H;
tsγ is a temperature-sensitive mutant in cistron G;
ts41D is a temperature-sensitive mutant in cistron F;
ts116 is a temperature-sensitive mutant in cistron B;
ts128 is a temperature-sensitive mutant in cistron A;
am3 is an amber mutant in cistron E;
am10 is an amber mutant in cistron D;
am16 is an amber mutant in cistron B;
am18 is an amber mutant in cistron A.

All the φX mutants were originally isolated and characterized in the laboratory of Dr R. L. Sinsheimer.

For phage titrations the agar layer method of Adams (1950) was used. Top agar contained 3 g beef extract, 5 g Peptone and 7 g agar/l. distilled water. Bottom agar contained 10 g Tryptone, 2·5 g NaCl, 2·5 g KCl, 6 ml. of 1 N-NaOH and 10 g agar/l. distilled water. After autoclaving, 1 ml. of sterile 1 M-$CaCl_2$ was added. All media were from Difco.

A distinction between the wild-type and *ts* mutants of φX can be made by plating under the following conditions: plating bacteria were log-phase *E. coli* C cells grown at 37°C in modified 3XD broth (0·9 g KH_2PO_4, 2·1 g Na_2HPO_4 1·0 g NH_4Cl, 1·0 ml. 1% gelatin solution, 15·0 g Casamino acids, 30·0 g glycerol, all made up to 1l. and autoclaved. After sterilization 10 ml. Mg SO_4 (6·1 g Mg $SO_4 \cdot 7H_2O$/100 ml.) and 0·3 ml. 1 M-$CaCl_2$ are added. The plates were shifted from 30 to 41°C 2·5 hr after titration of the phage. After incuba-

† When this work was in progress, Spatz & Trautner (1970) published experiments, in which they determined the replication direction of phage SPP1 using the same method.

tion for 4 hr at 41°C the plaques were counted. *ts* mutants produce tiny plaques and wild-type phage produces large plaques.

A distinction between the wild-type and amber mutants of ϕX can be made by plating under the following conditions. Plating bacteria were log-phase *E. coli* CR34-C416 cells grown in modified 3XD broth. A layer of top agar containing *E. coli* C is placed on the plates 15 min before titration. After incubation of the plates at 37°C for 6 hr, the wild-type produces clear plaques and the amber mutants turbid plaques.

(c) *Preparation of DNA*

Single-stranded DNA (plus strand) of bacteriophage ϕX174 (Jansz, Van Rotterdam & Cohen, 1966) and double-stranded ϕX174 DNA (Jansz, Pouwels & Schiphorst, 1966) were prepared as previously described. The closed circular duplex (component I) of double-stranded ϕX DNA was isolated in a pure form by means of a CsCl gradient containing ethidium bromide by the procedure of Radloff, Bauer & Vinograd (1967). The closed circular duplex was converted to the nicked circular duplex (component II) by pancreatic deoxyribonuclease as previously described (Jansz, Baas, Pouwels, Van Bruggen & Oldenziel, 1968).

The minus strand of ϕX DNA was obtained by heat denaturation for 3 min at 90°C in 0·01 M-Tris–0·001 M-EDTA, pH 8·5, of double-stranded ϕX DNA containing approximately equal quantities of component I and component II in the presence of the same amount of poly(U,G) (Baas & Jansz, 1971,1972).

(d) *Preparation of heteroduplex replicative form DNA*

Equal amounts (5 to 10 μg/ml.) of plus- and minus-strand were annealed at 65°C for 90 min in 2 × SSC (SSC is 0·15 M-NaCl–0·015 M-sodium citrate, pH 7·0).

After annealing the DNA solution, adjusted if necessary to 1·0 ml. with 2 × SSC, was passed through a MF14 filter. This filter retains single-stranded DNA whereas double-stranded DNA passes through (Jansz, Pouwels & Schiphorst, 1966). The filters were washed with 0·5 ml. 2 × SSC and the wash solution was added to the filtrate.

(e) *Biological activity of ϕX174 DNA*

DNA was incubated with spheroplasts of *E. coli* K58 at 30°C according to Guthrie & Sinsheimer (1960).

Single-burst experiments were performed as follows: 6 min after addition of DNA, the spheroplasts were diluted in nutrient broth containing 2% bovine serum albumin. From the appropriate dilution, 0·1 ml. was added to 0·5 ml. nutrient broth and incubated for 3 hr at 30°C. After incubation, the contents of the tubes were titrated for mature ϕX phage.

In most of the single-burst experiments, different dilutions of the infected spheroplasts were used. Dilutions in which the mean number of infected spheroplasts per test tube was more than 0·5 were discarded. The fraction of spheroplasts producing both genotypes was corrected, using the Poisson equation for the contribution of mixed bursts in tubes containing a spheroplast yielding wild-type and a spheroplast yielding mutant phage.

3. Results and Discussion

In previous papers (Baas & Jansz, 1971,1972) we have described the preparation and properties of heteroduplex ϕX RF which is composed of a plus- and minus-strand derived from ϕX phage of different genotype. Spheroplasts infected with heteroduplex RF yield three different kinds of bursts. The great majority of the infected spheroplasts produces a pure burst either of phage of the plus-strand or of the minus-strand genotype, and a small fraction of the infected spheroplasts produces phage of both genotypes. We have presented evidence that after infection heteroduplex RF is converted to homoduplex RF before replication. This repair process results in the production of pure bursts. In a small fraction of the spheroplasts, heteroduplex RF escapes this repair process, i.e. heteroduplex RF is replicated before the repair

system can work. The first cycle of semiconservative DNA replication then produces two homoduplexes, one of the plus-strand and one of the minus-strand genotype. In this case the infected spheroplast yields a mixed burst.

If the following assumptions are made:

(1) that the DNA replication of double-stranded ϕX DNA starts from a fixed point, the origin of DNA replication; and

(2) that transcription, translation and replication are not affected by the nature or position of a particular heteroduplex region,

then the only parameter in the competition between repair and replication is the distance between the origin of ϕX DNA replication and the heteroduplex region. Therefore, a heteroduplex region located near to the origin of ϕX DNA replication has a better chance of escaping the repair process than a heteroduplex region which is located near the terminus of ϕX DNA replication. As a consequence, spheroplasts, infected with heteroduplex RF, with a heteroduplex region located near the origin of DNA replication, produce more mixed bursts than spheroplasts infected with heteroduplex RF in which the heteroduplex region is located near the terminus of DNA replication.

To test this hypothesis, we have prepared a set of heteroduplex RF molecules, in which the minus strand was isolated from wild-type RF and the plus strand was obtained from temperature-sensitive or amber mutants of ϕX in different cistrons. The position of the mutants on the genetic map of ϕX is shown in Figure 1 (Benbow, Hutchison, Fabricant & Sinsheimer, 1971). In single-burst experiments under permissive conditions, the frequencies of the three kinds of bursts were determined for the different heteroduplex RF molecules (Table 1).

In Figure 2 the percentage of mixed bursts produced by the different heteroduplex RF molecules is plotted *versus* the distance on the genetic map of ϕX.

The results can be summarized as follows:

(1) There is a difference in the percentage of mixed bursts produced in spheroplasts infected with different heteroduplex RF molecules.

(2) The percentage of mixed bursts is correlated with the position of the heteroduplex region on the genetic map of ϕX in a linear way.

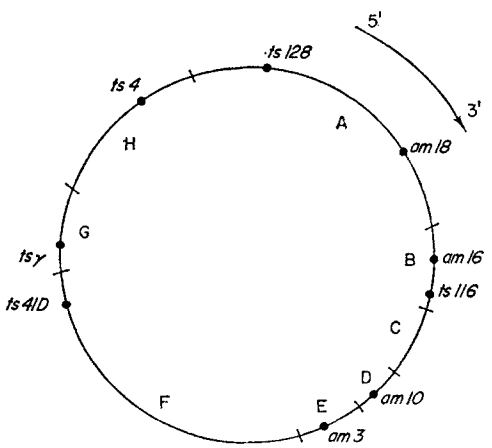

FIG. 1. Part of the genetic map of bacteriophage ϕX174. The map incorporates both two-factor and three-factor crosses (Benbow *et al.*, 1971).

TABLE 1

Single-burst experiments with different heteroduplex replicative form molecules

Heteroduplex	No. of bursts tested	No. of bursts with wild-type phage (minus strand)	No. of bursts with mutant phage (plus strand)	No. of bursts with both genotypes	Mean number of infected spheroplasts per test tube	Percentage mixed bursts (Poisson corrected)
ts128(+) ϕ_0(−)	76	29=38%	43=57%	4= 5%	0·094	4
ts4(+) ϕ_0(−)	67	18	41	8	0·22	7
		=35%	=55%	=10%		
	49	23	23	3	0·105	
tsy(+) ϕ_0(−)	98	33	49	16	0·40	
		=37%	=47%	=16%		10
	73	31	31	11	0.17	
ts41D(+) ϕ_0(−)	122	34=28%	61=50%	27=22%	0·31	17
am3(+) ϕ_0(−)	56	28	10	18	0·34	
	34	13=45%	10=26%	11=29%	0·15	26
	35	16	12	7	0·11	
am10(+) ϕ_0(−)	112	49	28	35	0·42	
		=46%	=22%	=32%		29
	97	46	18	33	0·25	
ts116(+) ϕ_0(−)	66	24	16	26	0·42	
	87	28=35%	31=29%	28=36%	0·36	32
	46	17	12	17	0·13	
am16(+) ϕ_0(−)	64	23	21	20	0·21	
		=30%	=39%	=31%		30
	44	9	21	14	0·105	
am18(+) ϕ_0(−)	95	38	23	34	0·50	
		=40%	=27%	=33%		27
	122	49	36	37	0·36	

Heteroduplex RF, containing a minus-strand derived from wild-type ϕX phage and a plus-strand obtained from different temperature-sensitive or amber mutants of ϕX phage, was added to spheroplasts of *E. coli* K58 (*su*$^+$). The spheroplasts were distributed to single-burst tubes and incubated for 3 hr at 30°C to permit lysis 6 min after the addition of heteroduplex RF. The resulting progeny phage were analysed with respect to the genetic marker.

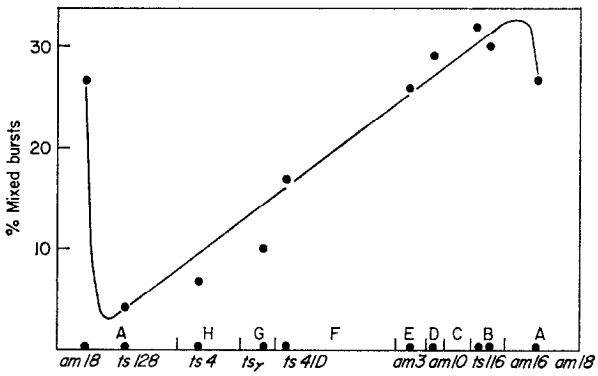

FIG. 2. The percentage of mixed bursts, produced at 30°C by infection of spheroplasts of *E. coli* K58 (*su*$^+$) with different heteroduplex RF, was determined in single-burst experiments (see Table 1). This percentage is plotted against the distance of the heteroduplex region on the genetic map of ϕX.

(3) There is a relation between the percentage of mixed bursts and the percentage of bursts yielding only phage of the minus-strand genotype.

From these results the following conclusions can be drawn. The replication of double-stranded ϕX DNA is unidirectional and clockwise with regard to the genetic map of ϕX. In this experiment the position of the origin can be deduced from a discontinuity in the plot of the percentage mixed bursts *versus* the genetic map of ϕX. As shown in Figure 2, this discontinuity is located near the position of *am18* on the genetic map.

Benbow *et al.* (1971) have found that the direction of translation in ϕX is clockwise with regard to the genetic map. The end of cistron A (the position of *am18*) and the beginning of cistron B is an attractive place for the origin of ϕX replication, because in a coupled transcription–translation system (Stent, 1966) the cistron A product, which is required for RF replication (Levine & Sinsheimer, 1968,1969*a*,*b*), could immediately induce a cycle of replication after it is synthesized. Also the origin of DNA replication in other systems, such as *E. coli* (Helmstetter, 1968; Pato & Glaser, 1968; Cerdá-Olmedo, Hanawalt & Guerola, 1968; Masters, 1970), λ (Schnöss & Inman, 1970; Stevens *et al.*, 1971) and T4 (Mosig, 1970) is located close to the genes that are involved in DNA replication.

It is interesting to note that the position of the origin of DNA replication at the end of cistron A coincides with a region of anomalously high recombination (Benbow *et al.*, 1971). This may be correlated with the nicking of the plus-strand at the origin of DNA replication as proposed in the rolling circle model of Dressler & Wolfson (1970).

In the preceding paper (Baas & Jansz, 1972), we have described the inhibition of the repair process of heteroduplex RF by treatment of spheroplasts of *E. coli* K12 *hcr*$^-$ by mitomycin C. The result of this inhibition is an increase of spheroplasts producing both genotypes, and an increase of spheroplasts producing only phage of the minus-strand genotype. It was shown that in mixed bursts the majority of the phage has the genotype of the minus strand. From these results, we concluded that the minus strand of ϕX is the master template in the RF replication, in agreement with the rolling circle model of Dressler & Wolfson (1970). Also, in the present experiments

the majority of the phage in mixed bursts has the genotype of the minus-strand. Heteroduplex RF with a heteroduplex region located near the origin of replication yields, in addition to a higher fraction of mixed bursts, a significantly higher fraction of pure bursts of the minus-strand genotype. The fact that a closer position of the heteroduplex region to the origin and mitomycin C treatment causes the same phenomena, indicates that the antibiotic exerts its effect by inhibiting repair rather than having an odd effect on RF replication.

From the translation direction (see above) of ϕX, the polarity of the plus-strand can be deduced. Because the minus-strand of ϕX is transcribed by RNA polymerase (Hayashi, Hayashi & Spiegelman, 1963), the polarity of the plus-strand is the same as in ϕX messenger-RNA. It is known that the translation process proceeds in the 5′→3′ direction, so it follows that the 5′→3′ direction of the plus-strand is clockwise with regard to the genetic map. Analysis of the fine structure and mode of linkage of the newly synthesized T4 DNA at the replication fork by Okazaki & Okazaki (1969) provided evidence that DNA on both strands is synthesized in the 5′→3′ direction. A polymerization of nucleotides in the opposite direction has never been found *in vivo* nor *in vitro*. So it is generally assumed that DNA is synthesized in the 5′→3′ direction. In the literature two different rolling circle models in which one strand remains circular and the other is elongated in the 5′→3′ direction for ϕX RF replication are proposed.

In the rolling circle model of Knippers, Whalley & Sinsheimer (1969), the parental plus-strand remains circular and the minus-strand is elongated. On the other hand, Dressler & Wolfson (1970) propose a rolling circle model in which the parental minus-strand remains circular and the plus-strand is elongated in the 5′→3′ direction. The replication direction of double-stranded ϕX DNA, as found in the present work, is in agreement with the model proposed by Dressler & Wolfson, since only an elongation of the plus strand in the 5′→3′ direction results in a ϕX DNA replication which is clockwise with regard to the genetic map.

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