RESTORATION OF THE BIOLOGICAL ACTIVITY OF IN VITRO SYNTHESIZED ϕX DNA BY TRANSFECTION OF *ung*$^-$ SPHEROPLASTS OR dUTPase TREATMENT

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1. Introduction

Knowledge of the complete nucleotide sequence of the genome of bacteriophage ϕX174 [1] and advancement in the synthesis of oligodeoxyribonucleotides by pure chemical means [2] has made it possible to study the relationship between the biological function of DNA and the nucleotide sequence by creating and studying preselected mutants. Oligodeoxyribonucleotides complementary to the wild-type DNA sequence of the viral strand except for a preselected mutation, can be synthesized and used as primers for DNA polymerase with wild-type viral DNA as template. After ligation and isolation of the closed circular heteroduplex DNA, a limited DNase I digestion, followed by poly(U·G)/CsCl centrifugation will yield the mutated complementary DNA strand in a pure form. This complementary DNA strand can then be used to transfect *Escherichia coli* spheroplasts to propagate the desired mutant (fig.1). Recent studies [3–5] using a similar approach have shown that it is possible to obtain 10–30% mutants by transfection with heteroduplex RFI DNA. In these studies known ϕX *am* mutants (*am*3 and *am*16) and its revertants were synthesized using wild-type viral strand and a mutated primer and vice versa. The *am* mutants and revertants could be easily selected for by different plating conditions. However, transfection with mutated single-stranded complementary strand DNA instead of heteroduplex RFI DNA is required, not only for the isolation of silent mutations for which no selection procedure is possible, but also for the detection of lethal mutations. This procedure should also eliminate heteroduplex repair.

Using this approach we want to study which nucleotides in ϕX DNA are essential for the origin function in ϕX DNA replication. We have shown [6–8] that ϕX DNA replication starts at nucleotide 4305 after nicking of the viral strand by the cistron A protein. A number of oligodeoxyribonucleotides complementary to the origin region, each containing one preselected mutation will be used as primers for in vitro DNA synthesis in the described system. The complementary strands with the different primers will be isolated and tested in the spheroplast system for their biological activity. Complementary strands with a mutation in the origin region should yield viable phage particles only, if the mutated nucleotide is non-essential for a functional origin of DNA replication; it will yield no phage (lethal mutation) if the changed nucleotide is essential for origin function.

We report here a study of the isolation and the biological activity of in vitro synthesized complementary ϕX DNA using a synthetic wild-type oligonucleotide primer. The biological activity of this DNA in the spheroplast system is very low. However the biological activity can be restored by transfection of spheroplasts made from an *Escherichia coli* strain which lacks the enzyme uracil-*N*-glucosidase (*ung*$^-$) or by treatment of the dNTP mix prior to the polymerisation reaction with puri-

Abbreviations: RFI, replicative form DNA with both strands closed; RFII, replicative form DNA with one or more discontinuities in either strand; Relaxed RF DNA contains no superhelical turns.

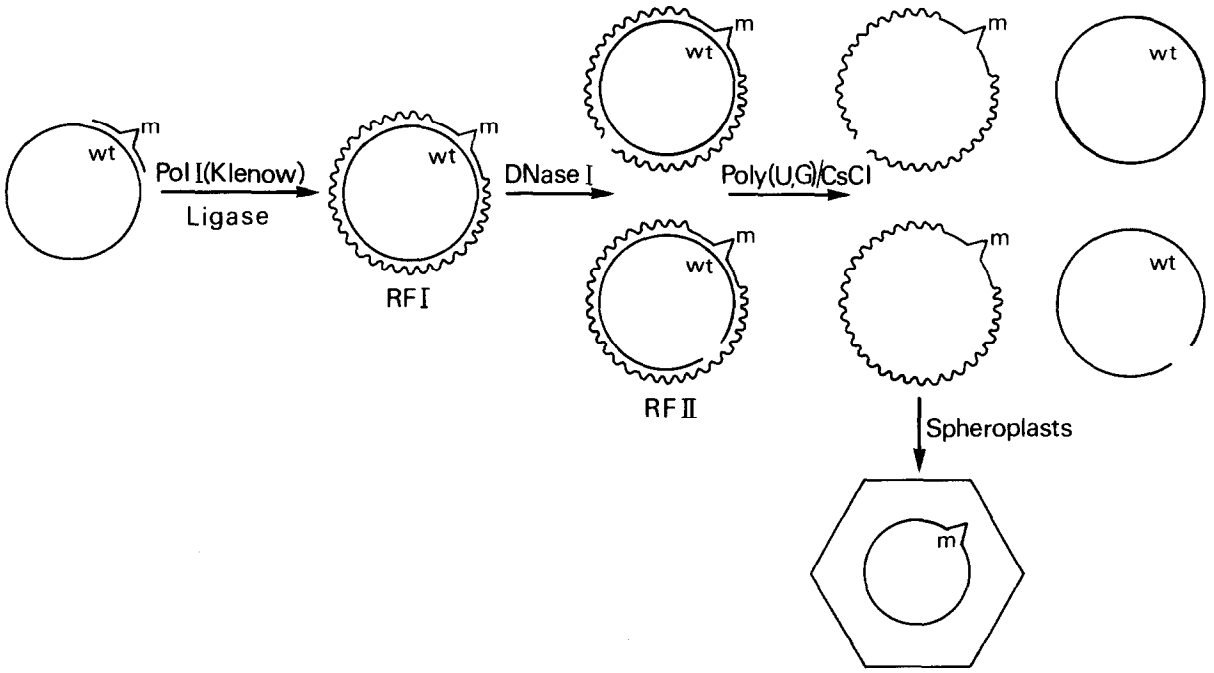

Fig.1. Scheme for the synthesis and isolation of specific mutants of bacteriophage ϕX174. A synthetic oligodeoxyribonucleotide complementary to the wild-type (wt) DNA sequence of the viral strand, except for one preselected mutation (m) is used as a primer for *Escherichia coli* DNA polymerase I (Klenow fragment) in the presence of *Escherichia coli* DNA ligase. Heteroduplex RFI DNA is isolated and treated with DNase I to convert ~50% of the RFI into RFII DNA. After poly (U·G)/CsCl gradient centrifugation the mutant complementary strand is isolated and propagated in the spheroplast system. RFI DNA made in vitro contains no superhelical turns. ϕX linears are not infectious in a spheroplast assay. The synthesis of the different deoxyhexadecamers will be published elsewhere.

fied dUTPase. This suggests strongly that dUTP residues present as contaminants in our reaction mixture are incorporated in the complementary DNA strands. After transfection of *Escherichia coli ung*$^+$ spheroplasts with uracil containing single-stranded DNA, the enzyme uracil-*N*-glucosidase excises the uracil from the DNA and other nucleases then cleave the circle [9–13] resulting in loss of the biological activity.

2. Materials and methods

2.1. *DNA polymerisation reaction and isolation of covalently closed RFI DNA*

Wild-type ϕX DNA (2 μg) and 0.18 μg oligodeoxyribonucleotide complementary to the wild-type ϕX DNA sequence (4299–4314) [1] was annealed for 20 h at 20°C in 60 μl *Pol* I buffer (31 mM KPO_4; pH 7.4). Then 150 μl 'dNTP mix' in *Pol* I buffer containing 20 μCi d[^{3}H]TTP (15–30 Ci/mM, Amersham), 0.075 mM dGTP, dATP, dCTP and dTTP, 10 mM $MgCl_2$, 12 mM DTT and 37 μM NAD^+ was added to the DNA solution. The polymerisation reaction was started by addition of 2.3 units *Escherichia coli* DNA polymerase I, Klenow fragment (Boehringer) and 2 units *Escherichia coli* DNA ligase (Biolabs) at 4°C. The amount of RFI DNA synthesized is higher, if the reaction is carried out at low temperature, probably because of reduction of the displacement synthesis. After 17 h at 4°C the reaction was stopped by adding 100 μl Tris–EDTA buffer (0.05–0.005 M; pH 8.0) and two consecutive ethanol precipitations were carried out to remove most of the non-incorporated d[^{3}H]TTP. The DNA was dissolved in 8.35 ml Tris–EDTA buffer and 0.25 ml propidium diiodide (4 mg/ml), and 7.70 g solid CsCl were added to the solution. The CsCl gradient was spun for 64 h at 10°C in a Beckman 50Ti rotor at 38 000 rev./min. The closed circular RFI DNA (fractions 8–10, fig.2) was freed from propidium diiodide by passing through a Dowex column (50 W × 8, 200–400 mesh).

2.2. *dUTPase treatment of the dNTP mix*

dUTPase (0.5 μl) kindly provided by Dr Shlomai (1.32×10^6 U/ml; spec. act. 19×10^6 U/mg) [23] was added to 150 μl 'dNTP mix' and this was incubated first 10 min at 0°C and then during 15 min at 30°C. Then the dNTP mix was transfered to the DNA solution and after 15 min at 4°C *Escherichia coli* DNA polymerase I and *Escherichia coli* DNA ligase were added.

2.3. *Limited DNase I digestion and isolation of complementary φX DNA*

To closed circular RFI DNA in 0.01 M Tris (pH 8.0) $MgCl_2$ was added to 0.01 M and DNase I (Nutritional Biochem. Co.) to 0.0005 μg/ml and this was incubated during 7 min at 20°C. The reaction was stopped by addition of sodium citrate to 0.012 M. Poly(U·G)/CsCl gradient centrifugation for the separation of viral and complementary φX DNA after denaturation for 3 min at 100°C was performed as in [6,14].

2.4. *Preparation and infection of spheroplasts*

DNA was assayed on spheroplasts of *Escherichia coli* K58 (*ung*$^+$, *sup*$^+$) and *Escherichia coli* K58 (*ung*$^-$, *sup*$^+$) [15,16]. The latter strain was constructed by P_1 transduction from strain BW 212 (*dut*-11, *ung*-1) [17].

3. Results and discussion

In preliminary experiments to obtain suitable conditions for the synthesis of RFI DNA, we used as primers the restriction enzyme fragment *Alu*8 [18], obtained from wild-type RF DNA and a 16 nucleotide long oligodeoxyribonucleotide with a sequence completely complementary to the wild-type origin of φX (4299–4314). In both cases routinely >50% of the incorporated radioactivity sedimented in a propidium diiodide–CsCl gradient to the left of the supertwisted RFI DNA marker (fig.2). After BND-cellulose chromatography of this material >85% of the radioactivity was found in the double-stranded fraction. This indicates that the great majority of this material consists of relaxed RFI DNA and not of rolling circle-type molecules [19]. This RFI DNA had a normal biological activity in the spheroplast system. However, after DNase I treatment and poly(U·G)/CsCl gradient centrifugation the biological activity of the isolated complementary strand was very low (fig.3, table 1). Two possible explanations for the biological inactivity of the complementary DNA strand can be envisaged:

(1) The complementary DNA strand preparation contains no circular molecules (circularity of the φX genome is required for the biological activity);
(2) The biological activity could have been destroyed by mistakes caused by a faulty incorporation during the DNA polymerisation reaction.

Possibility (1) could be ruled out by centrifugational analyses which indicated the presence of circular complementary strands in our DNA preparation (not shown). For possibility (2) dUTP seems a likely

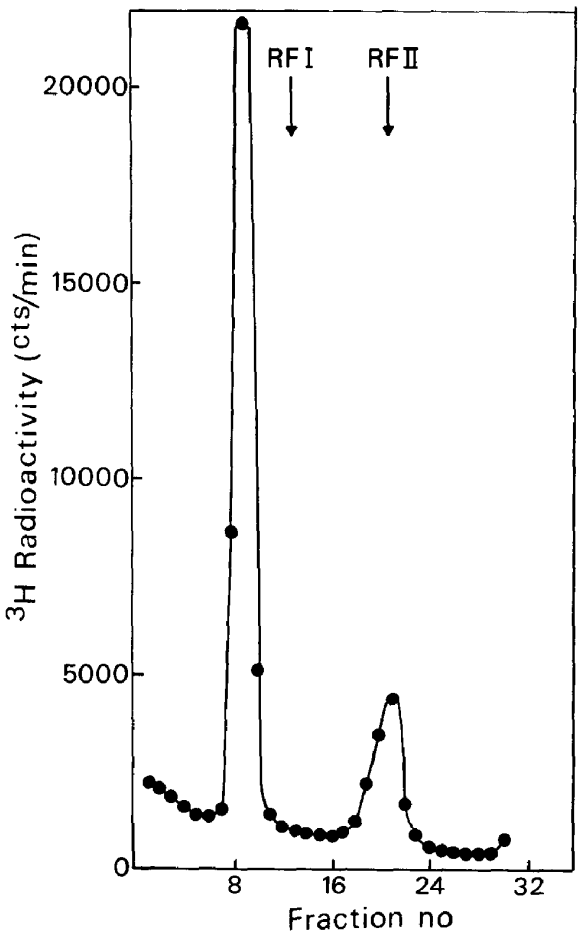

Fig.2. Propidium diiodide–CsCl gradient centrifugation of the product of the DNA polymerisation reaction. The arrows indicate the position of supertwisted RFI DNA and of RFII DNA. (•———•) ^{3}H radioactivity (cpm); 10 μl aliquots of the fractionated gradient were counted. The direction of the sedimentation is from right to left.

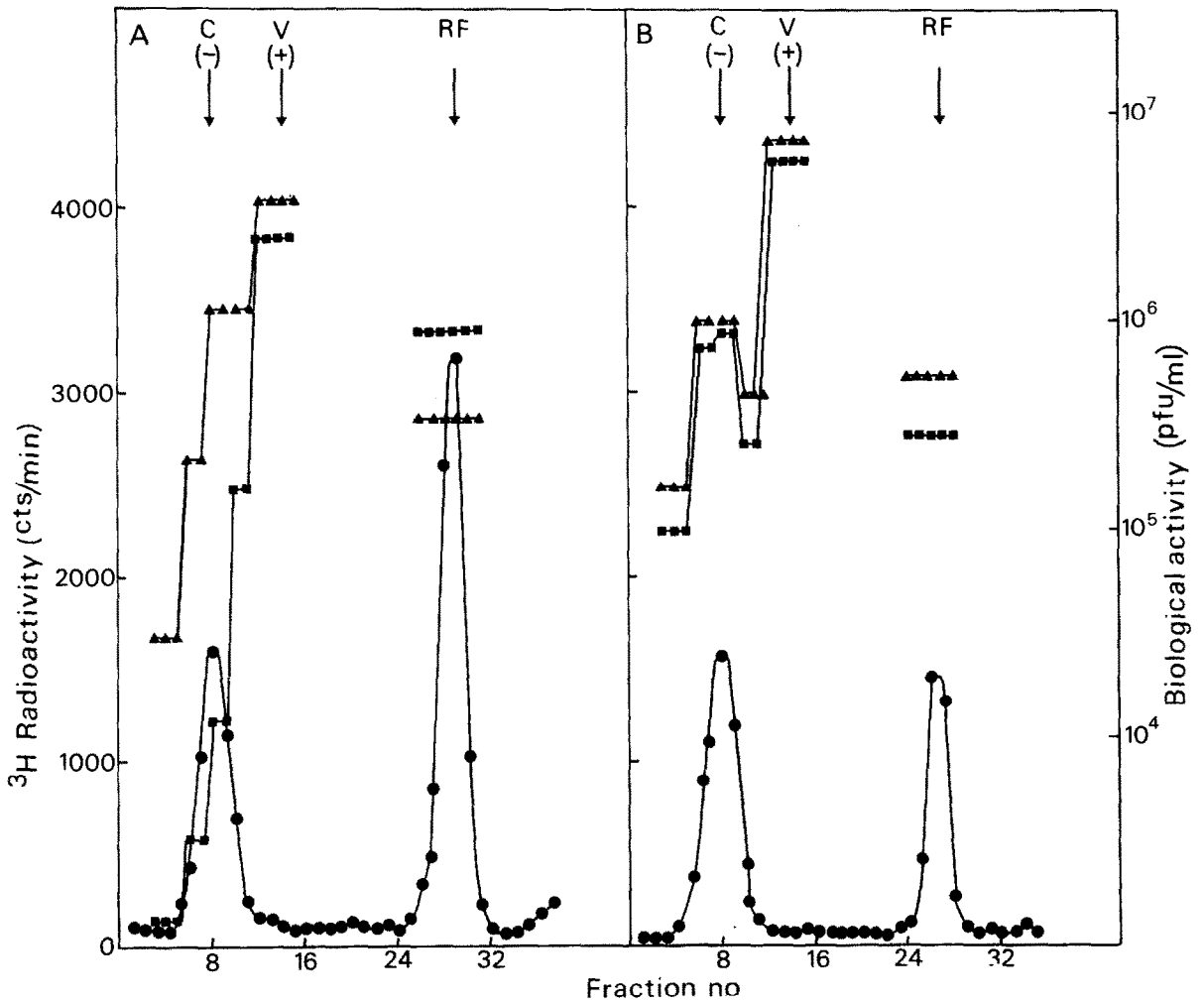

Fig.3. Poly(U·G/)/CsCl gradient centrifugation of closed circular RF DNA, synthesized without (A) and after dUTPase treatment (B), after limited digestion with DNase I. Some fractions of the poly(U-G)/CsCl gradient were combined (see table 1) and tested after dialysis against 0.01 M Tris (pH 8.0) in the spheroplast system. Spheroplasts were prepared both from *Escherichia coli* K58 (*ung*$^-$, *sup*$^+$) and *Escherichia coli* K58 (*ung*$^+$, *sup*$^+$). The arrows indicate the positions of the complementary strand, the viral strand and RF DNA. (●——●) ^{3}H radioactivity (cpm/min); 5 μl aliquots of the different fractions were counted. (▲——▲) Biological activity (p.f.u./ml) in spheroplasts of *Escherichia coli* K58 (*ung*$^-$). (■——■) Biological activity (p.f.u./ml) in spheroplasts of *Escherichia coli* K58 (*ung*$^+$). The direction of sedimentation is from right to left.

candidate because recent studies have shown that dUTP is incorporated efficiently into DNA both in vivo [17,20,21] and in vitro [22–24]. After transfection of spheroplasts with uracil containing single-stranded DNA the enzyme uracil *N*-glucosidase may then initiate the degradation of the circle and therefore destroy its biological activity [9–13]. In RFI DNA with one uracil containing strand, repair processes would preserve the biological activity of the molecule. To test the possibility that dUTP incorporation was responsible for the biological inactivity, the transfection was carried out in spheroplasts of *Escherichia coli* lacking the enzyme uracil-*N*-glucosidase (*ung*$^-$). In this system the biological activity of in vitro synthesized complementary DNA strand was completely restored (fig.3, table 1). To prove that the inactivity of the complementary DNA strand in *ung*$^+$ spheroplasts was due to the incorporation of dUTP, ϕX RFI DNA was also made by incubation of our reaction mixture with purified dUTPase prior to the poly-

Table 1
Plaque forming units/ml of different fractions of the poly(U·G)/CsCl gradient of φX RF DNA, synthesized without (A) and after (B) dUTPase treatment, in spheroplasts of *Escherichia coli* K58 (*ung*$^+$) and K58 (*ung*$^-$)

Fractions of the poly(U·G)/ CsCl gradient	−dUTPase		+dUTPase	
	K58 *ung*$^+$	K58 *ung*$^-$	K58 *ung*$^+$	K58 *ung*$^-$
3–5	1.4×10^3	3.5×10^4	1.0×10^5	1.7×10^5
6,7	3.6×10^3	2.3×10^5	7.2×10^5	1.0×10^6
8,9 (complementary DNA strand)[a]	1.4×10^4	1.1×10^6	9.5×10^5	1.0×10^6
10,11	1.6×10^5	1.1×10^6	2.7×10^5	4.1×10^5
12–15 (viral DNA strand)	2.3×10^6	3.5×10^6	6.7×10^6	8.0×10^6
26–31 (A) and 24–28 (B) (RF DNA)	9.0×10^5	3.3×10^5	3.1×10^5	5.8×10^5

[a] The efficiency of infection of the complementary strand is ~5-times less than that of the viral strand [14]

merisation reaction. From the RFI DNA synthesized in that way, complementary DNA strands were isolated and tested in *ung*$^+$ and *ung*$^-$ spheroplasts (fig.3, table 1). The complementary DNA strands were fully active in the spheroplast system regardless of the functioning of the *ung* gene.

These results indicate, that within *ung*$^+$ spheroplasts the attack of uracil residues by the enzyme uracil-*N*-glucosidase occurs faster than the conversion of the complementary strand to a double-stranded RF DNA. dUTP can be generated either by spontaneous or enzymatic deamination of dCTP. We do not know the amount of dUTP residues incorporated/ φX circle. As discussed [23], it is difficult to identify the source of dUTP at the presumed order of 10^{-8} M. We have tested in our system several different commercial enzyme preparations (*Pol* I and ligase) and also several, freshly prepared dNTP solutions with essentially the same result. The biological activity of in vitro synthesized φX DNA in [25] must be the result of a lower dUTP incorporation caused by different experimental conditions.

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