J. Mol. Biol. (1987) 198, 51–61

Mutational Analysis of the Bacteriophage φX174 Replication Origin

P. D. Baas

Institute of Molecular Biology and Medical Biotechnology and Laboratory for Physiological Chemistry State University of Utrecht, Padualaan 8 3584 CH Utrecht, The Netherlands

(Received 13 April 1987, and in revised form 15 July 1987)

Bacteriophage φX174 mutants within the 30 base-pair replication origin were constructed using oligodeoxynucleotide-directed mutagenesis. A total of 18 viable base substitution mutants at 13 different positions within the origin region were obtained. The majority of these ori mutants have a plaque morphology and burst size comparable to that of wild-type φX174. Two φX174 ori mutants with a reduced growth ability spontaneously acquired additional mutations that enhanced the growth rate. The additional mutation was located at the same site as the original mutation or was located in the N-terminal part of the gene *A* protein. This latter secondary mutation is responsible for a better binding and/or recognition of the gene *A* protein to the mutated origin. In a Darwinian experiment wild-type φX174 outgrows all φX174 ori mutants, indicating the superiority of the wild-type ori sequence for the reproduction of bacteriophage φX174.

Insertions and deletions were constructed at different positions within the φX174 replication origin cloned in a plasmid. Small insertions and deletions in the A+T-rich spacer region do not inhibit φX174 gene *A* protein cleavage *in vitro*, but severely impair packaging of single-stranded plasmid DNA in viral coats.

1. Introduction

The origin of rolling circle DNA replication of bacteriophage φX174 consists of a DNA sequence of 30 bp† (Fig. 1), which is found highly conserved among the members of the isometric phage group (Godson *et al.*, 1978; Heidekamp *et al.*, 1980, 1982; Lau & Spencer, 1985). The origin is located within the structural gene for the initiator protein, the gene *A* protein (Baas *et al.*, 1976). The origin is of paramount importance for the life cycle of bacteriophage φX174. It is involved in various protein–DNA interactions during initiation, elongation and termination of rolling circle DNA replication and also during DNA packaging, which is strongly coupled to rolling circle DNA replication late in the infection cycle of bacteriophage φX174.

During initiation of rolling circle DNA replication φX174 gene *A* protein cleaves the phosphodiester bond between nucleotides 4305 (G) and 4306 (A) of the viral strand of the parental RF DNA (Langeveld *et al.*, 1978; Sanger *et al.*, 1978). One of the tyrosyl residues of the gene *A* protein in position 343 or 347 forms a covalent ester bond with the phosphate group of the adenylic acid residue at position 4306 of the DNA sequence (Van Mansfeld *et al.*, 1986). Complex formation between the *rep* protein and the gene *A* protein covalently bound to the origin leads to unwinding of the DNA duplex. This unwinding provides an entry site for DNA polymerase III holoenzyme. During DNA replication the 5′ end of the displaced viral strand travels along with the replication fork in a looped rolling circle. At the end of the replication cycle the gene *A* protein, still covalently bound at the 5′ end of the parental viral strand, cleaves the regenerated origin. In a cleavage–ligation reaction the 3′ and 5′ ends of the parental viral strand are sealed to form a circle, whereas the gene *A* protein is transferred to the newly synthesized viral strand (Eisenberg *et al.*, 1977, 1978). Late in infection the association of the prohead to the RFII–gene *A* protein–*rep* protein complex at the origin, which is mediated by φX174 gene *C* protein, results in a coupling of rolling circle

† Abbreviations used: bp, base-pair(s); RFI, replicative form, double-stranded DNA with both strands closed containing superhelical turns; RFII, replicative form, double-stranded DNA with one or more discontinuities in either strand; RFIV, replicative form, double-stranded DNA with both strands closed containing no superhelical turns; SSB, single-stranded DNA binding protein of *Escherichia coli*.

0022-2836/87/210051-11 $03.00/0

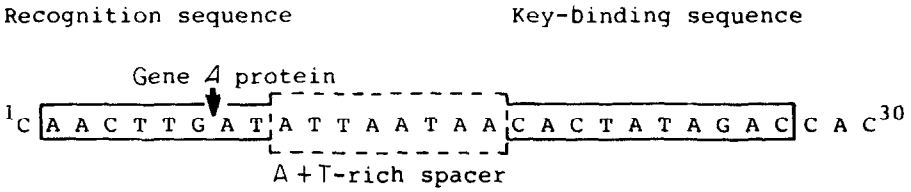

Figure 1. The ϕX174 replication origin, nucleotides 4299–4328 of the ϕX174 DNA sequence (Sanger *et al.*, 1978), with its different functional domains. The arrow indicates the gene *A* protein cleavage site. The complete 30 bp origin is both necessary and sufficient for rolling circle DNA replication coupled to DNA packaging *in vivo*.

DNA replication and DNA packaging (Aoyama & Hayashi, 1986). In this way the displaced viral strand is replicated directly into the phage prohead (Koths & Dressler, 1980; Aoyama *et al.*, 1983).

Various studies *in vivo* and *in vitro* have been carried out to establish the boundaries and the nucleotide sequence requirements in the origin region for different aspects during the replication cycle (for a review, see Baas, 1985). These studies have led to the postulation of the following model for interaction of ϕX174 gene *A* protein with the replication origin during initiation of rolling circle DNA replication (Baas *et al.*, 1981*a*). The ϕX174 replication origin consists of different functional domains: a recognition and key-binding sequence of gene *A* protein, which are separated by an A+T-rich spacer region (Fig. 1). In superhelical RFI DNA gene *A* protein binds first to the 3′ part of the origin region, the key-binding sequence. This binding induces local denaturation of the origin region which is facilitated by the A+T-rich region. Then the 5′ part of the origin region becomes exposed in a single-stranded state and is recognized and cleaved by gene *A* protein. The resulting RFII–gene *A* protein complex is the substrate for rolling circle DNA replication.

This paper describes the result of a detailed mutational analysis of the ϕX174 replication origin. ϕX174 mutant phages have been obtained using oligodeoxynucleotide-directed mutagenesis and have been tested for viability. Also deletions and insertions in the ϕX174 replication origin cloned in plasmid DNA have been constructed. These plasmids were tested *in vitro* as substrates for gene *A* protein cleavage and *in vivo* as substrates in the ϕX174- and G4-mediated transduction systems. Part of the results of this study have been published in a review paper on DNA replication of single-stranded *Escherichia coli* DNA phages (Baas, 1985).

2. Materials and Methods

(a) *Phage and bacterial strains*

Escherichia coli strain C122 was used as the normal host for plating and propagation of wild-type ϕX174 and ϕX174 ori mutants. *E. coli* K58 *ung*$^-$ was used as the host in the spheroplast assay for ϕX174 DNA (Baas *et al.*, 1980). *E. coli* HF4704 (Thy$^-$, *uvrA*, $\phi X^S 174$ Sup$^-$; Benbow *et al.*, 1974) was used as the recipient strain in the transformation assay (Dagert & Ehrlich, 1979). *E. coli* HF4712 (Sup$^+$) (Benbow *et al.*, 1974) was used to grow ϕX174 *am3 ts27* (Weisbeek *et al.*, 1973) and G4 *amE2* (Borrias *et al.*, 1979). The transduction experiments with ϕX174 and G4 were performed as described (Fluit *et al.*, 1985). Marker rescue experiments were performed as follows: 4 ng of ori-18 DNA, equivalent to 10^6 phages in a spheroplast assay, was mixed with a 10-fold excess of a particular restriction DNA fragment in 150 μl of 0·01 M-Tris·HCl (pH 8·0). The mixture was heated for 2 min in a boiling water bath and subsequently incubated for 1 h at 60 °C. This mixture was then analyzed in the spheroplast assay. The preparation of ori-18 DNA, incubated without restriction DNA fragments, yields 1% large sized plaques in the spheroplast assay.

(b) *Synthetic oligodeoxynucleotides*

Various oligodeoxynucleotides corresponding to different parts of the viral (+) and complementary (−) strand of ϕX174 DNA were obtained by chemical synthesis (Arentzen *et al.*, 1979; De Rooij *et al.*, 1979; Marugg *et al.*, 1982, 1983). The different oligodeoxynucleotides used in this study are listed in Table 1. The wild-type oligodeoxynucleotides of the complementary strand type (−) were used as a control in the mutagenesis experiments, as a probe for the detection of ϕX174 ori mutants, and as a primer in the sequence analysis according to the chain termination method (Sanger *et al.*, 1977, 1980). Oligodeoxynucleotides identical (+) and complementary (−) to the first 16 nucleotides of the ϕX174 replication origin were used for the construction of insertions in the ϕX174 replication origin. Oligodeoxynucleotides complementary to parts of the ϕX174 replication origin with preselected nucleotide changes were used to induce ϕX174 ori mutants. The nucleotide sequences of the oligodeoxynucleotides, except for the mixed oligodeoxynucleotides, were confirmed by sequence analysis according to the chemical modification technique (Maxam & Gilbert, 1980) or by the mobility shift method as described (Van Mansfeld *et al.*, 1980). The 5′-OH ends of the oligodeoxynucleotides were phosphorylated with non-radioactive ATP or [γ-^{32}P]ATP by phage T4 polynucleotide kinase according to the protocol of Maxam & Gilbert (1980).

(c) *Construction, selection and characterization of ϕX174 ori mutants*

Heteroduplex ϕX174 RFIV DNA was constructed and isolated using wild-type ϕX174 DNA as a template and a mutant oligodeoxynucleotide as primer for DNA polymerase I, Klenow fragment (Boehringer, Mannheim) essentially as described (Baas *et al.*, 1980, 1981*b*). In later experiments the reactions were carried out at 15 °C instead of 4 °C and T4 DNA ligase was used for closure of the complementary strand instead of *E. coli* DNA ligase.

Table 1

Oligodeoxynucleotides used for the construction and analysis of φX174 ori mutants

DNA sequence†		Location on the φX174 map‡	Genotype†
CAACTTGATATTAATA		1–16 (+)	Wild-type
TATTAATATCAAGTTG		1–16 (−)	Wild-type
CTATAGTGTTATTAA		11–25 (−)	Wild-type
GTGGTCTATAGTGTTATTAA		11–30 (−)	Wild-type
CGGTGGTCTATAGTGTT		16–32 (−)	Wild-type
CTATAGTGTTG*TTAA		11–25 (−)	Mutant-15
CTATAGTA*TTATTAA		11–25 (−)	Mutant-18
CTATT*GTGTTATTAA		11–25 (−)	Mutant-21
CA*ATAGTGTTATTAA		11–25 (−)	Mutant-24
GTGA*TCTATAGTGTTATTAA		11–30 (−)	Mutant-27
TC*TTAATATCAAGTTG		1–16 (−)	Mutant-15
AXAGXGXXAXXAAXAT§		8–23 (−)	Mixed mutant primer 10, 13, 14, 16, 17, 19, 22
CGYTYGTZTATAYTGTT‖		16–32 (−)	Mixed mutant primer 20, 25, 28, 30
CAAGCATTGGGGATTG	φX174:	4089–4104 (−)	Wild-type
ACCGTCTTCTCGTTCT	φX174:	4360–4375 (−)	Wild-type

† Nucleotides marked with an asterisk are changed nucleotides. The position of the changed nucleotide(s) in the φX174 replication origin is indicated.

‡ 1–16 (+) indicates the first 16 nucleotides of the viral strand of the φX174 replication origin. 11–30 (−) indicates nucleotides complementary to nucleotides 11 to 30 of the φX174 replication origin. φX174: 4089–4104 (−) indicates nucleotides complementary to nucleotides nr 4089–4104 of the φX174 map (Sanger *et al.*, 1978).

§ X = 70% T, 10% G, 10% C, 10% A.

‖ Y = 50% G, 16·67% C, 16·67% T, 16·67% A; Z = 50% C, 16·67% G, 16·67% T, 16·67% A.

Spheroplasts of *E. coli* K58 *ung*$^-$ were transfected either by RFIV DNA or by the isolated complementary strand. A variation in the percentage of mutant phage obtained was observed in different experiments depending amongst others on the oligodeoxynucleotide used. For primers with a unique nucleotide change the percentage of mutants varied from 5 to 20% for RFIV DNA and from 10 to 70% for the isolated complementary strand. The percentage of mutants obtained with RFIV DNA synthesized with the mixed oligodeoxynucleotide primers was 1% and 0·1% for oligodeoxynucleotide 8–23 (−) and 16–32 (−), respectively. This low yield of mutants may be partly explained by preferential hybridization to the template of the wild-type primer present in the oligodeoxynucleotide mixture. To avoid isolation and characterization of the same mutant different transfection assays obtained from the same DNA sample were analyzed. φX174 ori mutants were selected by means of a lack of hybridization of a wild-type oligodeoxynucleotide to DNA of plaques on nitrocellulose filters. The hybridization solution (Wallace *et al.*, 1979) contained 1×10^6 to 2×10^6 disints/min per ml end-labeled [^{32}P]oligodeoxynucleotide. After hybridization for at least 16 h the filters were washed 5 times for 10 min in 2 × SSC (SSC is 0·15 M-sodium chloride, 0·015 M-sodium citrate) at the same temperature as used for the hybridization. DNA hybridization was performed with oligodeoxynucleotides 1–16 (−), 11–25 (−), 11–30 (−), 16–32 (−) at 30 °C, 28 °C, 50 °C and 44 °C, respectively.

The DNA sequence of putative ori mutants was determined by sequence analysis according to the chain termination method (Sanger *et al.*, 1977, 1980). Wild-type ^{32}P end-labeled oligodeoxynucleotide complementary to nucleotides 4360–4375 (−) of the φX174 DNA sequence was used as a primer on φX174 DNA. In this way the DNA sequence throughout the whole origin region was established. The burst size of the ori mutants was determined in a one-step growth experiment. Synchronization of the infection was accomplished by addition of KCN prior to the infection (Denhardt & Sinsheimer, 1965).

(d) *Construction, selection and characterization of deletion and insertion mutants in the φX174 replication origin*

Deletions and insertions were constructed in the φX174 replication origin using the vectors pACYC177 (Chang & Cohen, 1978) and pBR322 (Sutcliffe, 1978). Restriction enzyme digestions and ligation of DNA fragments with T4 DNA ligase were essentially as described by Maniatis *et al.* (1982). Characterization and selection of the plasmid DNAs was performed by colony hybridization (Fluit *et al.*, 1985), antibiotic resistance, determination of the length of the plasmid DNA and restriction enzyme analysis of plasmid DNA isolated according to the procedure of Birnboim & Doly (1979). With a wild-type origin probe using appropriate hybridization conditions a differential hybridization response can be obtained with plasmid DNA containing the wild-type origin sequence (wild-type response) and with plasmid DNA containing one (mutant response) or more (no response) mutations in the origin region. Finally the DNA sequence of the mutated origin on the plasmid DNA was determined according to the chain termination method using ^{32}P end-labeled oligodeoxynucleotide 4360–4375 (−) as a primer as described by Wallace *et al.* (1981).

p-ori-del3 was obtained by ligation of the *Taq*I 2

restriction DNA fragment (ϕX174 nr 4304–5355) of ϕX174 ori-6 in the *Cla*I restriction site of pACYC177. The orientation of the *Taq*I restriction fragment is opposite to the transcription direction of the kanamycin resistance gene. p-ori-del3·4 was obtained by ligation of the *Taq*I 2 restriction DNA fragment (ϕX174 nr 4304–5355) of ϕX174 ori-6 in the *Cla*I restriction site of pBR322. The orientation of the *Taq*I restriction fragment is in the transcription direction of the tetracycline resistance gene. p-ori-12 was obtained by ligation of the *Taq*I 2 restriction DNA fragment (ϕX174 nr 4180–5355) of ϕX174 ori-12 into the *Cla*I restriction site of pBR322. The orientation of the *Taq*I restriction fragment is in the transcription direction of the tetracycline resistance gene. Subsequently, the *Hin*dIII–*Bam*HI fragment of the resulting plasmid was removed. After cleavage with the restriction enzymes *Bam*HI and *Hin*dIII the resulting sticky-end restriction sites were made blunt-end by incubation with DNA polymerase I Klenow fragment in the presence of the 4 dNTPs. In this way the *Eco*RV restriction site of pBR322 was removed. p-ori-12 thus contains one *Eco*RV restriction site in the mutated ϕX174 replication origin. p-ori-del9 and p-ori-del10 were constructed from p-ori-12 after cleavage with the restriction enzyme *Eco*RV and religation. Transformants which give no hybridization response with wild-type oligodeoxynucleotide 1–16 (−) were selected and sequenced. In this way a deletion of one nucleotide at either side of the *Eco*RV restriction site was obtained. p-ori-ins17, +11 was obtained by ligation of the double-stranded synthetic DNA corresponding to the first 16 bp of the ϕX174 replication origin into the filled-in *Cla*I restriction site of p-ori-del3·4. Individual transformants with a positive hybridization response to the wild-type 1–16 (−) oligodeoxynucleotide were picked up. DNA sequence analysis of individual transformants was performed to verify the DNA sequence and to identify clones with an insertion in the right orientation. p-ori-ins17, +7 was obtained in a similar way by ligating the double-stranded synthetic ori fragment into the filled-in *Cla*I and *Eco*RV site of p-ori-12.

(e) *Cleavage with ϕX174 gene* A *protein*

ϕX174 gene *A* protein was purified by affinity chromatography using single-stranded DNA cellulose and heparin–Sepharose columns as described by Langeveld *et al.* (1980). The gene *A* protein fraction also contained some A* protein. Gene *A* protein incubation was carried out as described by Heidekamp *et al.* (1981).

3. Results

(a) *Oligodeoxynucleotide-directed mutagenesis using unique oligodeoxynucleotide primers*

In a previous study I have used oligodeoxynucleotides which could alter the nucleotides at positions 6, 7, 8, 9 and 12 of the origin region of bacteriophage ϕX174 DNA (Baas *et al.*, 1981*b*). T→C substitutions at positions 6 and 12, respectively, yielded viable origin mutants, whereas nucleotide changes at position 7, 8 or 9 seemed to be lethal. This kind of mutational analysis is complicated in ϕX174 because the origin is located in the gene coding for the gene *A* protein (Baas *et al.*, 1976). Therefore, further analysis of the nucleotide specificity of the ϕX174 replication origin, using unique oligodeoxynucleotides was restricted to positions 15, 18, 21, 24 and 27 of the origin. Primers were designed in such a way that the amino acid sequence of the gene *A* protein of the corresponding ori mutants remains unchanged (Table 1). Viable ori mutants were obtained with base changes at positions 15, 18 and 21 of the origin, whereas no viable mutants could be isolated using oligodeoxynucleotides with base changes at position 24 or 27 (Table 2).

(b) *Oligodeoxynucleotide-directed mutagenesis using mixed oligodeoxynucleotide primers*

The results obtained with the mutational analysis of the replication origin indicate that within the origin region certain nucleotide changes at some positions are tolerated. Failure to obtain viable ϕX174 ori mutants with certain oligodeoxynucleotide primers on the other hand indicates that at certain positions within the origin specific nucleotides are required for proper origin function.

Studies *in vitro* using purified gene *A* protein have shown that single-stranded DNA containing the first ten nucleotides of the origin region is cleaved by gene *A* protein (Van Mansfeld *et al.*, 1980). This sequence is called the recognition sequence of gene *A* protein. The nucleotide sequence requirements of the recognition sequence have been studied *in vitro* using purified gene *A* protein and oligodeoxynucleotides with defined sequences (Van Mansfeld *et al.*, 1984). These studies have led to the following consensus sequence:

```
   A     T ↓ T
N  ACT   G   A  N
   T     C     G
1                10
```

for the recognition sequence of gene *A* protein. In order to study in more detail the nucleotide specificity of the A+T-rich spacer and the key-binding sequence, two mixed oligodeoxynucleotide primers were used to induce ϕX174 ori mutants. The first oligodeoxynucleotide primer is complementary to nucleotides 8–23 (−) of the origin. The nucleotides incorporated complementary to positions 10, 13, 14, 16, 17, 19 and 22 consisted of a mixture of 70% T (wild-type) and 10% A, 10% G and 10% C. The primer mixture synthesized in this way contains approximately 8% wild-type primer. A total of 21% of the primer mixture contains a single mutation (21 different ones) and the remaining part (approximately 70%) contains double and multiple mutations. The second primer is complementary to nucleotides 16–32 (−) of the origin. The nucleotides incorporated complementary to positions 20, 25, 28 and 30 consisted of a mixture of 50% of the wild-type nucleotide and 16·67% each of the remaining nucleotides. In this case the final mixture contains 6% wild-type primer and a total of 25% has a single mutation (12 different ones) and the remaining 69% consists of double and multiple mutations. ϕX174 ori mutants

Table 2

Bacteriophage φX174 ori mutants constructed by oligodeoxynucleotide-directed mutagenesis

Mutant position	Nucleotide change	Amino acid change in gene *A* protein	Burst size‡
ori-6	T → C	None	Normal 90%
ori-10	A → T	Ile → Phe	Reduced 50%
ori-11 (St-1,α_3)†	T → A	Ile → Asn	—
ori-12	T → C	None	Normal 100%
ori-13	A → T	Asn → Tyr	Normal 105%
ori-14 (St-1, α_3)†	A → G	Asn → Ser	—
ori-14·1	A → T	Asn → Ile	Normal 100%
ori-14·2	A → C	Asn → Thr	Normal 100%
ori-14·3	A → G	Asn → Ser	Normal 105%
ori-15·1	T → C	None	Reduced 15%
ori-15·2	T → A	Asn → Lys	Normal 100%
ori-17	A → C	Asn → Thr	Normal 95%
ori-17,19	17A → T	Asn → Ile	Reduced 50%
	19 A → G	Thr → Ala	Reduced 10%
ori-18	C → T	None	Reduced 10%
ori-19·1	A → G	Thr → Ala	Normal 95%
ori-19·2	A → C	Thr → Pro	Normal 95%
ori-20·1	C → T	Thr → Ile	Normal 100%
ori-20·2	C → G	Thr → Ser	Normal 100%
ori-21	T → A	None	Normal 100%
ori-22	A → C	Ile → Leu	Normal 95%

No viable mutants could be isolated using oligodeoxynucleotide-directed mutagenesis with primers containing the following base changes.

7	G → A	Asp → Asn
8	A → G or T	Asp → Gly or Val
9	T → G	Asp → Glu
15	T → G	Asn → Lys
24	A → T	None
27	C → T	None

† Nucleotide changes found in the origin of bacteriophage St-1 and α_3 are included in this Table (Heidekamp *et al.*, 1980, 1982).

‡ Burst size of the mutants was compared to the burst size of wild-type φX174 determined in the same experiment. Value of the burst size of wild-type φX174 varied in different experiments between 120 and 180. ori mutants with a reduced burst size, ori-10, ori-15·1, ori-18 and ori-17·19, give small plaques on titration with *E. coli C*.

induced by both primers have a changed amino acid sequence of the gene *A* protein. Transfection of spheroplasts with circular covalently closed RF DNA synthesized using the first primer 8–23 (−) yielded a total of 0·3% to 1% mutant plaques in three different experiments. With the second primer the yield of mutants in one experiment was 0·1%. DNA sequencing of the mutants showed that with the first primer nine different single φX174 ori mutants were obtained and one double mutant (Table 2). The mutations were located at six of the possible seven sites. No φX174 ori mutants were isolated with a nucleotide change at position 16. DNA sequence analysis of four independently isolated mutants obtained using the second mixed primer showed only nucleotide changes at position 20. One mutant contained a C→T change, and the other three a C→G substitution. No φX174 ori mutants were isolated with a nucleotide change at position 25, 28 or 30.

(c) *Growth characteristics of φX174 ori mutants*

The procedure for the mutant induction (spheroplast transfection and phage titration) has been carried out at 30 °C in order to avoid loss of possible temperature-sensitive ori mutants. Further growth and titration of the mutants at 37 °C and 41 °C indicated that no temperature-sensitive mutants were isolated. The plaque morphology of the majority of the ori mutants was indistinguishable from that of wild-type φX174. However, ori-10, ori-15·1, ori-18 and the double mutant ori-17, 19 form smaller plaques than wild-type φX174 on titration with *E. coli* C. From all the mutants the burst size and the duration of the infection cycle was determined in a one-step growth experiment. The burst size of the majority of the ori mutants was comparable with that of the control wild-type φX174 (Table 2). Also no differences in the duration of the infection cycle were observed. However, the

burst size of the mutants with a small plaque morphology was significantly lower than that of wild-type ϕX174. The burst size of ori-10, ori-15·1, ori-18 and ori-17,19 was 50%, 15%, 10% and 50%, respectively, of that of the wild-type control. The small plaque morphology and the low phage yield after one growth cycle indicate that the nucleotide substitutions present in the origin of these mutants influence the viability of these phages.

(d) *Wild-type ϕX174 outgrows ori mutants in a Darwinian experiment*

As described in the preceding section no significant difference in plaque morphology, in burst size and in duration of the infection cycle between the majority of the ori mutants and wild-type ϕX174 could be detected. However, small differences in burst size may be unnoticed in a one-step growth experiment. Therefore, a Darwinian type of experiment was performed. In this experiment wild-type ϕX174 was mixed with an equal amount of 11 different ori mutants (ori-6, ori-10, ori-12, ori-13, ori-14·1, ori-14·2, ori-15·1, ori-15·2, ori-17, ori-18 and ori-21). This mixture was used to infect an *E. coli* culture (10^8 bacteria/ml) with a multiplicity of infection of 10^{-4}. Three hours after infection the culture was chilled and phage were isolated. This phage preparation was used to infect a new fresh *E. coli* culture under the same conditions. Six consecutive infection cycles were performed in this way.

The phage obtained after each cycle were characterized by plaque morphology and hybridization response to an oligodeoxynucleotide complementary to the first 16 nucleotides (1–16 (−),16) of the origin region and to an oligodeoxynucleotide (15) complementary to nucleotides 11–25 (−) of the origin region. After one cycle the ori mutants with a reduced growth capacity could not be detected. After six cycles the percentage of wild-type phage starting at 14% has reached a value of 83%. DNA obtained after the last cycle from four different plaques that showed a mutant hybridization response with both oligodeoxynucleotides (16^-, 15^-), and DNA obtained from four different plaques that showed a mutant hybridization response only with the pentadecamer (16^+, 15^-) was sequenced. The four mutants with the 16^+ 15^- hybridization response were all ori-21, whereas two of the phages of the 16^- 15^- class were ori-13, one ori-14·1 and the other turned out to be ori-14·2. The gradual reduction in the percentage of mutant phage observed after each growth cycle indicates that wild-type ϕX174 finally outgrows all the ϕX174 ori mutants. This indicates that the wild-type ori sequence is superior to that of the mutant sequences.

(e) *Spontaneous secondary mutations improve the growth rate of impaired ϕX174 ori mutants*

ori-15·1 is a ϕX174 ori mutant characterized by small plaque morphology and low burst size. The T→C base change of this mutant has no influence

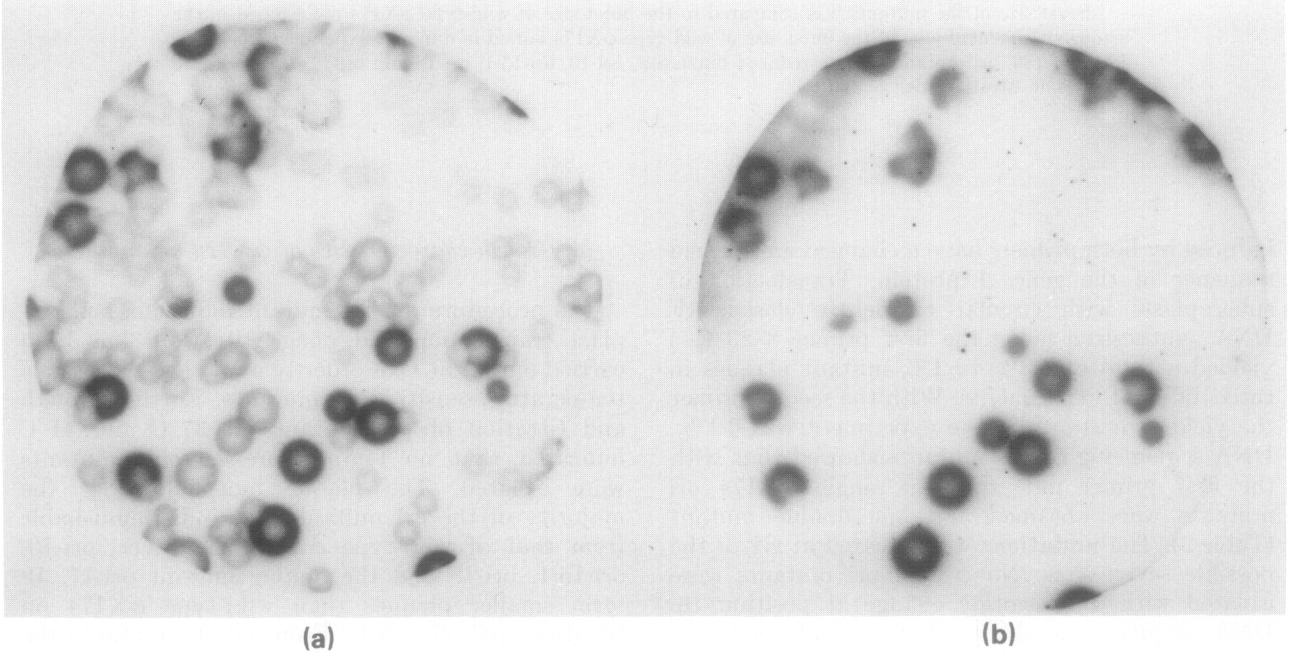

Figure 2. Bacteriophage ϕX174 ori-15·1 was grown on *E. coli* C for 2 cycles starting with low multiplicity of infection. Plaques of the final lysate were transferred to nitrocellulose filters and hybridized with oligodeoxynucleotide 11–25 (−) as a probe. (a) Hybridization and washing carried out at 22°C; (b) hybridization and washing carried out at 30°C. Wild-type ϕX174 shows a strong hybridization response at both temperatures. ori-15·1 and ori-15·2 show a weak hybridization response at 22°C and no hybridization signal at 30°C. The small mutant plaques at 22°C contain ori-15·1; the large mutant plaques contain ori-15·2.

on the amino acid sequence of the gene *A* protein. Further growth of this mutant (one or more cycles starting with a low multiplicity of infection) yields a phage lysate which contains phage producing normal sized φX174 plaques also. Figure 2 shows a hybridization experiment of such a lysate with an oligodeoxynucleotide complementary to nucleotides 11–25 (−) of the origin region. The small plaques (ori-15·1) show a mutant hybridization response, whereas the larger-sized plaques show a mutant or a wild-type hybridization response. DNA sequence analysis of a small plaque lysate confirmed the original T→C substitution at position 15 of the origin sequence. DNA sequencing of a large plaque lysate with wild-type hybridization response showed the wild-type ori sequence. DNA sequence analysis of a large plaque lysate with mutant hybridization response, however, showed an A residue at position 15 of the origin region (ori-15·2). This nucleotide change results in an asparagine to lysine substitution of the corresponding amino acid residue of the gene *A* protein. The ori-15·2 mutant has a burst size comparable to that of wild-type φX174. DNA sequence analysis of six different phage mutants isolated in the same way in three separate experiments showed the same base substitution in position 15 of the origin region. Apparently during DNA replication of ori-15·1 occasionally at position 15 of the origin region instead of a C(+) or G(−) an A(+) or T(−) (ori-15·2), or a T(+) or A(−) (wild-type φX174) residue is incorporated. The greater viability of ori-15·2 and wild-type φX174 compared to ori-15·1 explains why these phage are picked up so easily after further propagation of the ori-15·1 mutant. A G residue at position 15 was never found. A G residue in this position would also change, like in ori-15·2, the corresponding asparagine residue of gene *A* protein into a lysine residue. Mutant induction with an oligodeoxynucleotide designed especially for the purpose to induce a T→G substitution in this position also failed. These experiments, therefore, showed that at position 15 of the origin a T and an A residue yields a viable φX174 phage. A C residue in this position results in a φX174 phage with a strongly reduced replication capacity, whereas a T→G substitution at this position is lethal for the phage.

The same type of analysis was performed with the other φX174 mutants with a reduced burst size, ori-10 and ori-18. With ori-10 all the larger-sized plaques analyzed gave a wild-type hybridization response upon hybridization with an oligodeoxynucleotide complementary to the first 16 nucleotides, 1–16 (−), of the origin. With ori-18 the greater majority of the large plaques turned out to be wild-type φX174. However, two large-sized plaques were obtained, which showed a mutant hybridization response with the oligodeoxynucleotide complementary to nucleotides 11–25 (−) of the origin sequence. DNA sequence analysis of these mutants showed that the original C→T substitution at position 18 of the origin region was maintained. Further analysis of these mutants showed that they were identical. The mutant will be designated as ori-18.II. The burst size of the mutant amounted to 35% of that of wild-type phage. The burst size of the original ori-18 mutant amounted to 10% of that of wild-type phage. These results indicated that the ori-18 mutant had acquired spontaneously a secondary mutation, which compensated partly for the low viability of the mutant phage. In order to locate the position of the secondary mutation marker rescue experiments were performed. Therefore, RF DNA of ori-18.II was cleaved with several restriction enzymes. Different, isolated restriction DNA fragments were hybridized to ori-18 single-stranded viral DNA, and this partially double-stranded DNA was used to transfect spheroplasts of *E. coli* K58 (*ung*$^-$). Results of several transfection experiments are shown in Table 3. DNA sequencing of ori-18.II using a primer complementary to nucleotides 1–16 (−) of the φX174 origin region and a primer complementary to nucleotides 4089–4104 (−) showed, that ori-18.II contained a C→T substitution at position 4057 of the φX174 DNA sequence. This nucleotide change results in an amino acid change of an alanine residue into a valine residue in position 26 of gene *A* protein. ori-18 as well as our wild-type φX174 contains a C residue at that position. These results strongly suggest that the altered gene *A* protein has a higher affinity to the mutated origin sequence of ori-18 than wild-type gene *A* protein.

(f) *Insertion and deletion mutagenesis of the φX174 replication origin*

Mutational analysis of the φX174 replication origin and the results obtained by studying the cleavage of single- and double-stranded DNA by gene *A* protein *in vitro* have led to a model in which the replication origin is divided into different functional domains. The recognition and the key-binding sequence of the gene *A* protein are separated by the A+T-rich spacer sequence (Fig. 1). In order to study the effect of insertions and deletions, especially within the A+T-rich spacer region, on the origin function several recombinant DNA plasmids with different deletions and insertions were constructed. These plasmid DNAs were tested in two ways for origin function. Firstly, superhelical, covalently closed double-stranded DNA was tested *in vitro* as substrate for gene *A* protein. Secondly, the plasmids were tested in the φX174- and G4-mediated transduction system. In this *in vivo* assay *E. coli* containing the recombinant plasmid DNA was infected with bacteriophage φX174 or G4 and from the resulting lysate the phage titer and the number of *E. coli* cells that acquired the antibiotic resistance gene located on the recombinant plasmid DNA was determined. A functional ori sequence on the plasmid results in the production of transducing particles, e.g. single-stranded plasmid DNA

Table 3
Marker rescue experiment to localize secondary mutation in ori-18.11

Restriction DNA fragment	Location of the fragment in nucleotides on the ϕX174 map	% of large sized plaques obtained	Location of the mutation
*Bst*NI–*Xho*I	3500–162	36%	+
*Bst*NI–*Stu*I	3500–4486	30%	+
*Bst*NI–*Bst*N1	881–3500	5%	–
*Hae*III Z2	3128–4206	27%	+
*Hae*III Z3	4948–434	4%	–
*Hin*dII R4	3705–4200	16%	+

encapsidated into a phage coat. The transduction ratio, the ratio of particles containing single-stranded plasmid DNA to phage particles is a measure for the ori function of the tested plasmids.

For the construction of the different deletions and insertions DNA derived from ϕX174 ori-6 and ori-12 was used. The nucleotide changes in these mutant DNAs created a *Taq*I and an *Eco*RV restriction site, respectively, which have been exploited for the construction of different insertions and deletions. A plasmid DNA containing a deletion of nucleotide four of the origin sequence (p-ori-del4) was constructed by cloning the *Taq*I 2 restriction fragment of ori-6, nucleotides nr 4304–5355, in the *Cla*I site of pACYC177 in the anti-transcription direction of the kanamycin phosphotransferase gene. The presence of the sequence CA next to the *Cla*I restriction site in pACYC177 (Heidekamp *et al.*, 1981) results in an ori sequence with a deletion of one nucleotide at position 4. The construction of plasmids with a deletion of nucleotides 3 and 4 (p-ori-del3·4), a deletion of nucleotide 9 (p-ori-del9) and a deletion of nucleotide 10 (p-ori-del10) within the origin region has been described in Materials and Methods. Plasmid DNAs containing insertions of seven and eleven nucleotides, ATCAATA and CGATATTAATA, at position 17 of the origin region (p-ori-ins17,+7; p-ori-ins17,+11) were obtained by ligation of a synthetic DNA fragment corresponding to the first 16 bp the ori sequence into the filled-in *Cla*I and *Eco*RV restriction site of p-ori-12, and into the filled in *Cla*I restriction site of p-ori-del3·4, respectively.

The results of both ori tests carried out with the different mutated plasmid DNAs are shown in Table 4. Incubation of plasmid DNAs containing a mutated recognition sequence (p-ori-del3, p-ori-del3·4, p-ori-del9) with gene *A* protein showed no conversion of RFI DNA into RFII DNA (Fig. 3). However, p-ori-del10 DNA with a deletion just outside the recognition sequence is cleaved by gene *A* protein (Fig. 3). Also DNA of the insertion mutant p-ori-ins17,+7 is cleaved by gene *A* protein *in vitro*. However, when the insertion was increased from seven to eleven nucleotides, gene *A* protein could not cleave any more. Besides RFII, RFIV DNA was also observed after incubation of p-ori-ins17, +7 RFI DNA with gene *A* protein. Apparently insertion of seven nucleotides in the spacer region activates the ligation activity of gene *A* protein. This ligation activity, which is active during termination of DNA replication, is normally suppressed upon incubation of ϕX174 RFI DNA with gene *A* protein. Analysis of the cleaved mutated origin DNAs showed that ϕX174 gene *A* protein cleaves, as in the wild-type origin, the phosphodiester bond between the G and the A residue (data not shown).

The ori function of the mutants, expressed as the transduction ratio of the mutant DNA divided by the transduction ratio of a plasmid with a functional origin (p-ori-12), is severely impaired (Table 4). p-ori-del10 and p-ori-ins17,+7, whose DNA is cleaved by gene *A* protein *in vitro*, also show a low transduction ratio. Therefore, cleavage by gene *A* protein *in vitro* is not a sufficient

Table 4
ori function tests performed with deletions and insertions in the bacteriophage ϕX174 replication origin

Plasmid DNA	Gene *A* protein cleavage	Transduction ration plasmid DNA/ transduction ratio p-ori-12	
		ϕX174	G4
p-ori-del3	–	$<10^{-6}$	Not measured
p-ori-del3·4	–	$<10^{-6}$	1×10^{-4}
p-ori-del9	–	$<10^{-5}$	$<10^{-6}$
p-ori-del10	+	1×10^{-3}	8×10^{-4}
p-ori-ins17,+7	+	5×10^{-3}	6×10^{-2}
p-ori-ins17, +11	–	$<10^{-6}$	8×10^{-5}

The transduction ratios given are the average of 3 independent experiments.

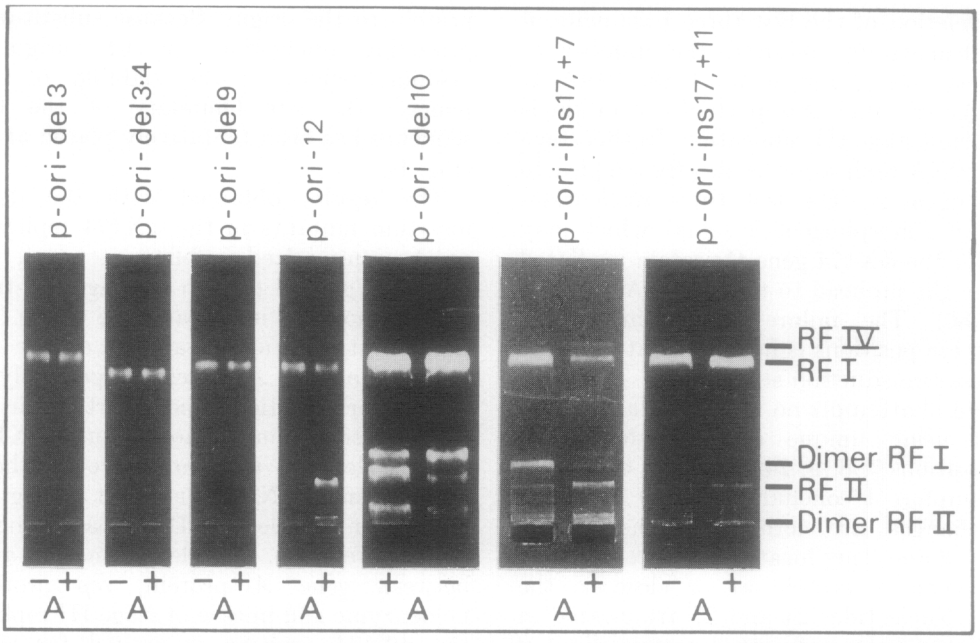

Figure 3. Analysis of φX174 gene *A* protein incubation of supercoiled, covalently closed plasmid DNA containing different insertions and deletions within the φX174 replication origin (+A). −A refers to the control experiment in which no φX174 gene *A* protein was added. p-ori-12 DNA was used as a positive control for φX174 gene *A* protein cleavage.

condition for rolling circle DNA replication coupled to DNA packaging *in vivo*. The relative differences between the φX174 and G4 transduction system are not understood.

Sequence analysis of plasmid DNA, isolated from single transducing colonies, obtained after infection of *E. coli* containing p-ori-del10, p-ori-ins17,+7 and p-ori-ins17,+11 showed the same ori sequence as in the original plasmid. This indicates that initiation and termination of DNA replication coupled to DNA packaging takes place in a normal way, albeit with reduced frequency. DNA sequence analysis of two transductants obtained after infection of p-ori-del3·4 with bacteriophage G4 showed the following sequences:

----G A A T G T A T T A G -
A T A T T A A T A A C A C T A T A -----
----C C G A A A A G T G C -
A T A T T A A T A A C A C T A T A-----

The underlined sequence is that of pBR322 nr 4269–4279 and pBR322 nr 4223–4233, respectively. The other part of the sequence starts with the A residue at the gene *A* protein cleavage site. These sequences probably arose by initiation of rolling circle DNA replication at the mutated origin and termination of DNA replication on the indicated pBR322 DNA sequence. The termination sites on pBR322 DNA show only remote sequence homology with the φX174 replication origin. So with very low frequency initiation and termination of rolling circle DNA replication takes place on heavily mutated ori sequences. These plasmid DNAs are not cleaved by gene *A* protein *in vitro* and no transducing particles have been obtained upon infection of *E. coli* strains carrying these plasmid with either φX174 or G4 phage.

4. Discussion

The results presented in this paper show that many nucleotide alterations in the φX174 replication origin are tolerated without important loss in viability of the resulting mutant phages. Nucleotide changes at 13 different positions within the 30 bp origin have yielded viable φX174 mutants. (The nucleotide change at position 11 found in phage St-1 and α3 is included in this number.) The majority of the mutants obtained are located in the A+T-rich spacer region (10–17). The majority of the mutants have a changed amino acid sequence of the gene *A* protein, indicating that this part of the gene *A* protein is not essential for the enzymatic reactions carried out by the gene *A* protein. The nature of the nucleotides in the A+T-rich spacer region is not completely unimportant for the viability of the phage as illustrated by the properties of the different mutants at positions 14 and 15.

Experiments using the mixed oligodeoxynucleotide primer complementary to nucleotides 16–32 (−) of the origin region, which contains nucleotide alterations at positions 20, 25, 28 and 30, only yielded four independent mutant phages. All four mutations were localized at position 20 of the origin. This suggests that base changes at the other positions may be lethal for the phage. Recent transduction studies using plasmid DNAs with partial origins and the complete 30 bp origin have

shown that deletion of the last three base-pairs of the origin dramatically reduced the number of transducing particles (Fluit *et al.*, 1985, 1986). It has been suggested that this part of the origin is important during stage III replication. In this stage rolling circle DNA replication is strictly coupled to phage morphogenesis. The last three nucleotides may be part of a morphogenetic signal, which may be required for the ϕX174 gene *C* protein-mediated attachment of the prohead to the DNA (Aoyama & Hayashi, 1986). The failure to obtain ϕX174 mutants at these positions is in agreement with the results of these transduction studies.

Despite several attempts no viable mutants could be obtained using unique oligodeoxynucleotide primers at positions 7, 8, 9, 24 and 27 of the origin (Table 2). Therefore I conclude that the indicated nucleotide changes are lethal for the phage. Nucleotides 7, 8 and 9 are located in the recognition sequence of gene *A* protein at or close to the cleavage site. Nucleotides 24 and 27 are located in the key-binding sequence of gene *A* protein. Fluit *et al.* (1984) have shown the important role of the C residue at position 27 of the origin in the ϕX174 gene *A* protein cleavage reaction.

The C residue at position 18 is also important during the recognition of the origin by the gene *A* protein. The C→T substitution at this position, which has no effect on the amino acid sequence of the gene *A* protein, results in the mutant phage ori-18 with a strongly reduced viability. A spontaneously occurring mutation in gene *A* increases the burst size of this mutant phage fourfold. The mutation results in an amino acid change, which is located in the N-terminal part of the gene *A* protein. This part of the gene *A* protein is responsible for the specificity of the cleavage of double-stranded DNA and not for the cleavage reaction *per se*. A* protein, which lacks the N-terminal third of the polypeptide chain of gene *A* protein, also cleaves and ligates single-stranded DNA, but is unable to cleave double-stranded DNA (Langeveld *et al.*, 1979, 1981; Eisenberg & Finer, 1980; Van Mansfeld *et al.*, 1982). The two tyrosyl residues involved in the cleavage ligation reaction of gene *A* protein have been determined in the C-terminal part of the gene *A* protein (Van Mansfeld *et al.*, 1986). The two tyrosyl residues lie only three amino acid residues apart and correspond to residues 343 and 347 of the gene *A* protein, respectively. The increased viability of ori-18.II *versus* ori-18 may be the result of a better recognition and binding of the mutated gene *A* protein to the mutated origin. The mutation changes the alanine residue at position 26 of the gene *A* protein into a valine residue. This residue is located in the sequence Lys-Pro-Val-Ile-Glu. The proximity of the proline and isoleucine residues makes it very unlikely that this part of the gene *A* protein has an α-helix conformation. Therefore the alanine to valine substitution may have important consequences for the conformation of the gene *A* protein, and therefore for the binding of gene *A* protein to the origin. Because substitutions in the preceding nucleotides of the origin have no profound effect on the viability of the mutant phages, the left boundary of the key-binding sequence has been tentatively placed at position 18 (Fig. 1).

The results obtained with the deletion and insertion mutants in the ϕX174 replication origin can be summarized as follows.

(1) The gene *A* protein cleavage and transduction studies show the important role of the C and the T residue at position 4 and 9 of the recognition sequence of gene *A* protein, respectively.

(2) Short deletions and insertions in the A+T-rich spacer region do not disturb ϕX174 gene *A* protein cleavage *in vitro*. However, the ability of these plasmid DNAs to support rolling circle DNA replication coupled to DNA packaging *in vivo* is severely reduced. Complex formation at the origin between gene *A* protein, *rep* protein, polIII holoenzyme and in case of stage III replication with the ϕX174 prohead is needed for rolling circle replication. Therefore the length of the A+T-rich spacer, as well as the nature of some nucleotides within this domain, may be important for the viability of the phage.

(3) The transducing particles obtained with p-ori-del3-4 (see above) indicate that occasionally DNA replication starts at a non-functional ori sequence. The termination site in the examples studied has almost no homology with the ϕX174 replication origin. These particles have been observed only in the G4 transduction system. The very low frequency at which they were obtained indicates that the appearance of these particles is more a curiosity of nature than a contribution to our understanding of ϕX174 DNA replication.

The overall picture emerging from this study is a ϕX174 replication origin in which a number of nucleotides fulfil an essential role during ϕX174 DNA replication. This can be either during ϕX174 gene *A* protein binding or cleavage of the origin, or formation of the replication fork or during phage morphogenesis. The data presented here, therefore, support the earlier proposed division of the origin into different functional domains. A number of nucleotides within the replication origin can be mutated without severe loss of replication ability of the resultant mutant phage. However, the replication ability of the wild-type ori sequence is superior to that of the mutants. This is clearly shown in the Darwinian experiment in which wild-type ϕX174 finally outgrows the ϕX174 ori mutants. The reduction of the burst size of the double mutation ori-17,19, compared to the normal burst size of single mutants at position 17 or 19, points also in the same direction. Although these nucleotides are not essential for the viability of ϕX174 phage, they apparently do contribute, as well as the other changeable nucleotides, to the fine tuning of the interaction of the proteins of the replication machinery with the bacteriophage ϕX174 replication origin.

I thank Mrs W. R. Teertstra, Mrs M. Hagenaar, Mrs H. Liewerink and Mrs T. Moen for their participation in several experiments. I thank Mr G. H. Veeneman and Dr J. H. Van Boom for the construction of the oligodeoxynucleotides. I thank Dr A. D. M. Van Mansfeld for a gift of φX174 gene *A* protein. I thank Dr H. S. Jansz for stimulating discussions during this work.

The research was supported in part by the Netherlands Organization for Chemical Research (S.O.N.) with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

References

Aoyama, A., Hamatake, R. K. & Hayashi, M. (1983). *Proc. Nat. Acad. Sci., U.S.A.* **80**, 4195–4199.

Aoyama, A. & Hayashi, M. (1986). *Cell,* **47**, 99–106.

Arentzen, R., Van Boeckel, C. A. A., Van der Marel, G. A. & Van Boom, J. H. (1979). *Synthesis*, 137–139.

Baas, P. D. (1985). *Biochim. Biophys. Acta,* **825**, 111–139.

Baas, P. D., Jansz, H. S. & Sinsheimer, R. L. (1976). *J. Mol. Biol.* **102**, 633–656.

Baas, P. D., Van Teeffelen, H. A. A. M., Teertstra, W. R., Jansz, H. S., Veeneman, G. H., Van der Marel, G. A. & Van Boom, J. H. (1980). *FEBS Letters,* **110**, 15–20.

Baas, P. D., Heidekamp, F., Van Mansfeld, A. D. M., Jansz, H. S., Van der Marel, G. A., Veeneman, G. H. & Van Boom, J. H. (1981*a*). In *The Initiation of DNA Replication: ICN-UCLA Symp. Mol. Cell Biol.* (Ray, D. S. & Fox, C. F., eds), vol. 22, pp.195–209, Academic Press, New York.

Baas, P. D., Teertstra, W. R., Van Mansfeld, A. D. M., Jansz, H. S., Van der Marel, G. A., Veeneman, G. H. & Van Boom, J. H. (1981*b*). *J. Mol. Biol.* **152**, 615–639.

Benbow, R. M., Zuccarelli, A. J., Davis, G. C. & Sinsheimer, R. L. (1974). *J. Virol.* **13**, 898–907.

Birnboim, H. C. & Doly, J. (1979). *Nucl. Acids Res.* **7**, 1513–1523.

Borrias, W. E., Hagenaar, M., Van den Brekel, R., Kuhlemeier, C. & Weisbeek, P. J. (1979). *J. Virol.* **31**, 288–298.

Chang, A. C. Y. & Cohen, S. N. (1978). *J. Bacteriol.* **134**, 1141–1156.

Dagert, M. & Ehrlich, S. D. (1979). *Gene,* **6**, 23–28.

Denhardt, D. T. & Sinsheimer, R. L. (1965). *J. Mol. Biol.* **12**, 641–646.

De Rooij, J. F. M., Wille-Hazeleger, G., Van Deursen, P. H., Serdijn, J. & Van Boom, J. H. (1979). *Recl. Trav. Chim. Pays-Bas*, **98**, 537–548.

Eisenberg, S. & Finer, M. (1980). *Nucl. Acids Res.* **8**, 5305–5315.

Eisenberg, S., Griffith, J. & Kornberg, A. (1977). *Proc. Nat. Acad. Sci., U.S.A.* **74**, 3198–3202.

Eisenberg, S., Scott, J. F. & Kornberg, A. (1978). In *The Single-stranded DNA Phages, Cold Spring Harbor Monograph series* (Denhardt, D. T., Dressler, D. H. & Ray, D. S., eds), pp. 287–302, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Fluit, A. C., Baas, P. D., Van Boom, J. H., Veeneman, G. H. & Jansz, H. S. (1984). *Nucl. Acids Res.* **12**, 6443–6454.

Fluit, A. C., Baas, P. D. & Jansz, H. S. (1985). *Eur. J. Biochem.* **149**, 579–584.

Fluit, A. C., Baas, P. D. & Jansz, H. S. (1986). *Virology,* **154**, 357–368.

Godson, G. N., Barrell, B. G., Staden, R. & Fiddes, J. C. (1978). *Nature (London),* **276**, 236–247.

Heidekamp, F., Langeveld, S. A., Baas, P. D. & Jansz, H. S. (1980). *Nucl. Acids Res.* **8**, 2009–2021.

Heidekamp, F., Baas, P. D., Van Boom, J. H., Veeneman, G. H., Zipursky, S. L. & Jansz, H. S. (1981). *Nucl. Acids Res.* **9**, 3335–3354.

Heidekamp, F., Baas, P. D. & Jansz, H. S. (1982). *J. Virol.* **42**, 91–99.

Koths, K. & Dressler, D. (1980). *J. Biol. Chem.* **255**, 4328–4338.

Langeveld, S. A., Van Mansfeld, A. D. M., Baas, P. D., Jansz, H. S., Van Arkel, G. A. & Weisbeek, P. J. (1978). *Nature (London),* **272**, 417–419.

Langeveld, S. A., Van Mansfeld, A. D. M., De Winter, J. & Weisbeek, P. J. (1979). *Nucl. Acids Res.* **7**, 2177–2188.

Langeveld, S. A., Van Arkel, G. A. & Weisbeek, P. J. (1980). *FEBS Letters*, **114**, 269–272.

Langeveld, S. A., Van Mansfeld, A. D. M., Van der Ende, A., Van de Pol, J. H., Van Arkel, G. A. & Weisbeek, P. J. (1981). *Nucl. Acids Res.* **9**, 545–562.

Lau, P. C. K. & Spencer, J. H. (1985). *Gene,* **40**, 273–284.

Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982). In *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Marugg, J. E., Van der Marel, G. A., de Vroom, E., Bosch, D. & Van Boom, J. H., (1982). *Recl. Trav. Chim. Pays-Bas*, **101**, 411–412.

Marugg, J. E., McLaughlin, L. W., Piel, N., Tromp, M., Van der Marel, G. A. & Van Boom, J. H. (1983). *Tetrahedron Letters,* **24**, 3989–3992.

Maxam, A. M. & Gilbert, W. (1980). *Methods Enzymol.* **65**, 499–560.

Sanger, F., Nicklen, S. & Coulson, A. R. (1977). *Proc. Nat. Acad. Sci.*, U.S.A. **74**, 5463–5467.

Sanger, F., Coulson, A. R., Friedmann, T., Air, G. M., Barrell, B. G., Brown, N. L., Fiddes, J. C., Hutchison, C. A. III, Slocombe, P. M. & Smith, M. (1978). *J. Mol. Biol.* **125**, 225–246.

Sanger, F., Coulson, A. R., Barrell, B. G., Smith, A. J. H. & Roe, B. A. (1980). *J. Mol. Biol.* **143**, 161–178.

Sutcliffe, J. G. (1978) *Cold Spring Harbor Symp. Quant. Biol.* **43**, 77–90.

Van Mansfeld, A. D. M., Langeveld, S. A., Baas, P. D., Jansz, H. S., Van der Marel, G. A., Veeneman, G. H. & Van Boom, J. H. (1980). *Nature (London),* **288**, 561–566.

Van Mansfeld, A. D. M., Van Teeffelen, H. A. A. M., Zandberg, J., Baas, P. D., Jansz, H. S., Veeneman, G. H. & Van Boom, J. H. (1982). *FEBS Letters*, **150**, 103–108.

Van Mansfeld, A. D. M., Baas, P. D. & Jansz, H. S. (1984). *Advan. Exp. Med. Biol.* **179**, 221–230.

Van Mansfeld, A. D. M., Van Teeffelen, H. A. A. M., Baas, P. D. & Jansz, H. S. (1986). *Nucl. Acids Res.* **14**, 4229–4238.

Wallace, R. B., Schaffer, J., Murphy, R. F., Bonner, J., Hirose, T. & Itakura, K. (1979). *Nucl. Acids Res.* **6**, 3543–3557.

Wallace, R. B., Johnson, M. J., Suggs, S. V., Miyoshi, K., Bhatt, R. & Itakura, K. (1981). *Gene,* **16**, 21–26.

Weisbeek, P. J., Van de Pol, J. H. & Van Arkel, G. A. (1973). *J. Virol.* **52**, 408–416.

Edited by A. Fersht