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Alteration of the ATG start codon of the A* protein of bacteriophage φX174 into an ATT codon yields a viable phage indicating that A* protein is not essential for φX174 reproduction

P.D. Baas, H. Liewerink, H.A.A.M. van Teeffelen, A.D.M. van Mansfeld, J.H. van Boom* and H.S. Jansz

Institute of Molecular Biology and Medical Biotechnology and Laboratory for Physiological Chemistry, State University of Utrecht, Padualaan 8, 3584 CH Utrecht and **Department of Organic Chemistry, State University of Leiden, Postbus 9502, 2300 RA Leiden, The Netherlands*

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Bacteriophage φX174 gene A encodes two proteins: the gene A protein and the smaller A* protein, which is synthesized from a translational start signal within the A gene in the same reading frame as the gene A protein. The gene A protein is involved in initiation, elongation and termination of rolling circle DNA replication. The role of the A* protein in the life cycle of φX174, however, is unknown. Using oligonucleotide-directed mutagenesis a viable φX174 mutant was constructed in which the ATG start codon of the A* protein was changed into an ATT codon. This mutant, φX-4499T, does not synthesize A* protein. The burst size of φX-4499T amounted to 50% of that of wild type φX174. This indicates that A* protein, although advantageous for phage reproduction, is not essential during the life cycle of bacteriophage φX174.

A* protein; Oligonucleotide-directed mutagenesis; ATG start codon; (Bacteriophage φX174)

1. INTRODUCTION

The function of the initiator gene A protein in the life cycle of bacteriophage φX174 has been elucidated by studying conditional lethal gene A mutants and by studying its role in in vitro φX174 DNA synthesizing systems (reviews [1-3]). φX174 rolling circle DNA replication is initiated by gene A protein cleavage of the viral strand of the parental RFI DNA at a unique site, the origin of replication [4]. During this cleavage reaction an ester bond is formed between either one of the tyrosyl residues in position 343 or 347 of the gene A protein and the phosphate of the adenylic acid residue (no. 4306 of the φX174 DNA sequence [5]) at the 5'-end of the cleavage site [6]. The covalently bound gene A protein participates in the replication complex during elongation and termination of DNA replication. At the end of a replication round gene A protein cleaves the regenerated origin and ligates the 3'- and 5'-end of the viral strand to form a cicle.

The role of the A* protein, which is synthesized from an internal translation start within gene A [7], during the life cycle of bacteriophage φX174 is less clear. Mutational analysis of the A* protein is

Correspondence address: 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

Abbreviations: RFI, replicative form DNA with both strands closed containing superhelical turns; ss, single-stranded; ds, double-stranded; c, circular

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complicated, because any A* mutation also results in the same mutation in the gene A protein.

A* protein has been purified and its properties have been studied extensively in vitro. A* protein binds strongly to ds- and ss-DNA [8]. It has retained nuclease activity on the ss-DNA of gene A protein, although with a more relaxed sequence specificity [9]. It also binds covalently to the 5′-end of the cleaved DNA and is able to transfer the attached DNA to a 3′-OH-terminated acceptor [10,11].

A* protein has lost the ability to cleave the viral strand of φXRFI at the origin. It is not required in the RF→ss (c) in vitro φX174 DNA synthesizing system or in the stage III in vitro replication system in which the synthesized viral strand DNA is directly packaged into viral proheads [12]. It inhibits φX174 DNA synthesis in vitro by aspecific binding to ds-DNA [13]. A* protein has been implicated in shutoff of host DNA synthesis [14,15]. Shutoff of host DNA synthesis occurred only in φX174 infections in which the A* protein was present. Recent cloning of the A* protein showed that the A* protein is lethal for the host cell [16]. Upon induction of A* protein synthesis, *Escherichia coli* DNA synthesis was inhibited.

In order to obtain more information about the role of φX174 gene A* protein during the life cycle of φX174 we have mutagenized the ATG start codon of the A* protein using a mixed oligonucleotide primer. We have obtained a viable φX174 mutant, which synthesizes no A* protein upon infection of *E. coli* C. This indicates that the A* protein is not essential for the reproduction of bacteriophage φX174.

2. MATERIALS AND METHODS

2.1. *Phage and bacterial strains*

E. coli strain C112 (*E. coli* C) was used as the normal host for plating and propagation of wild type φX174 and φX174 mutants. *E. coli* K58 *ung*$^-$, *sup*$^+$ [17] was used as the host in the spheroplast assay for φX174 DNA. φX174 *am*3 is a lysis defective φX174 mutant [18].

2.2. *Construction, isolation and characterization of φX174 mutant phage*

φX-4499T, a mutant phage with a G→T substitution at position 4499 of the φX174 DNA sequence, was constructed using two oligonucleotide primers on wild type φX174 DNA as a template essentially as described by Zoller and Smith [19]. The 5′-P mutagenic primer, GATTTXXYAGTGGAGG, in which X represents 25% of each nucleotide and Y 80% T (wild type) and 20% C, is complementary to nucleotides 4489–4504 of the φX174 DNA sequence. As wild type 5′-OH primers ACCGTCTTCTCGTTCT and CAAGCATTGGGGATTG complementary to nucleotides 4360–4375 and 4089–4104 of the φX174 DNA sequence, respectively, were used. Complementary strand φX174 DNA was synthesized at 15°C during 16 h by *E. coli* DNA polymerase I, Klenow fragment (Boehringer, Mannheim, FRG) in the presence of T_4 DNA ligase (Boehringer, Mannheim). After transfection of *E. coli* K58 *ung*$^-$, *sup*$^+$ spheroplasts with φX174 DNA the resulting plaques were screened for mutants by filter hybridization using the wild type oligonucleotide complementary to nucleotdies 4489–4504 of the φX174 sequence as probe. Hybridization and washing were performed at 35°C. The hybridization solution [20] contained $1–2\times10^6$ dpm/ml end labelled ^{32}P-oligonucleotide. After a second screening a mutant plaque was propagated in a 150 ml culture of *E. coli* C at a cell density of about 10^8/ml in TK broth (10 g Bacto Trypton/l, 5 g KCl/l, 10 mM $MgSO_4$, 2 mM $CaCl_2$). The DNA sequence of the mutant was determined by sequence analysis according to the chain termination method [21,22] using a wild type, ^{32}P-end labelled oligonucleotide complementary to nucleotides 4532–4547 of the φX174 DNA sequence as a primer.

2.3. *Determination of the burst size of bacteriophage φX174*

E. coli C was grown in TK broth to a density of 3×10^8 cells/ml. To 0.85 ml of *E. coli* 0.1 ml of 0.03 M KCN was added to synchronize the subsequent infection. After 10 min *E. coli* C was infected with 1×10^8 phage suspended in 0.05 ml TK broth. 10 min later the culture was diluted 10^4 in prewarmed TK broth and incubated under vigorous shaking at 37°C. Samples at different time intervals were taken and the number of infective centers was determined by titration. The burst size of the phage was calculated by dividing the average number of infected centers after lysis by

the average number of infected centers before lysis of the *E. coli* vells.

2.4. *Detection of φX174 gene A protein and φX174 A* protein in φX174 infected cells*

E. coli C was grown in TK broth to a density of 5×10^8 cells/ml. The cells were infected with φX174 wild type, φX174 *am*3 or φX-4499T phages at a multiplicity of infection of 5. Samples of 10 ml were taken from the different cultures just before infection and at 10, 30 and 60 min after infection. The samples were rapidly cooled and centrifuged for 15 min at 10000 rpm at 0°C in a Sorvall SS34 rotor. The pellets were resuspended in 1 ml of 100 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM EDTA, spun down, resuspended in 1 ml of 100 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM EDTA, 5 mM β-mercaptoethanol and stored at −70°C. After thawing the cells were lysed by the addition of 10 μl solution of lysozyme (10 mg/ml) in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA and incubated for 30 min at 4°C and 2 min at 37°C. Then 250 μl of 5 M NaCl were added and the suspensions were mixed gently for 30 min at 0°C. The samples were centrifuged for 30 min at 16000 rpm at 0°C in a Sorvall SS34 rotor. The supernatants (lysates) were transferred to Eppendorf tubes and used in the following assay. 4 μl of the above lysates were incubated with 0.03 pmol of the internally ^{32}P-labelled oligonucleotide CAACTTG[^{32}P]ATATTAATAAC (approx. 50000 cpm) in a volume of 16 μl as described before [6]. The incubations were stopped by the addition of 2 μl of 0.1 M EDTA, pH 8.0. Then 14 μl of 5 mM Na-phosphate, pH 7.0, 1% SDS, 30 mM dithiothreitol, 10% glycerol, 0.05% bromophenol blue were added, the samples were heated for 2 min at 100°C and subjected to electrophoresis on a SDS-polyacrylamide gel (20%) as described by Laemmli [23]. After electrophoresis the radioactive material was visualized by autoradiography. The gels corresponding to the position of the gene A protein-oligonucleotide and A* protein-oligonucleotide complexes were cut out and the Čerenkow radiation was determined in a liquid scintillation counter.

3. RESULTS

Mutation of the ATG start codon of φX174 A* protein in such a way that initiation of A* protein synthesis is inhibited may provide insight into the function of the A* protein during reproduction of bacteriophage φX174. If it is possible to create a viable φX174 mutant, which does not synthesize A* protein, the conclusion that A* protein is not essential for φX174 reproduction is justified. However, the fact that any mutation in the ATG start codon of the A* protein will also change the methionine residue in position 173 of the gene A protein further complicates the approach. If this part of the gene A protein is important for the function of the gene A protein, substitution of this methionine residue may result in an inactive gene A protein. Therefore failure to obtain a viable φX174 mutant with an altered ATG start codon of the A* protein may be explained in either one or two ways, namely that the A* protein is essential for φX174 reproduction or that the presence of a methionine residue in position 173 of the gene A protein is important for the function of the gene A protein.

A mixed oligonucleotide, GATTTXXYAGTGGAGG (X represents 25% of each nucleotide and Y 80% T, wild type, and 20% G, respectively) complementary to nucleotides 4489–4504 of the φX174 DNA sequence, was synthesized to induce φX174 mutants with an altered ATG start codon of the A* protein. By using this oligonucleotide as a mutagenic agent the ATG start codon of the A* protein may be changed into any other triplet except a triplet with a G or T in the first position. These nucleotides were deliberately omitted during the synthesis of the oligonucleotide, because it is known that GTG and TTG may be used as initiation codons for protein synthesis [24]. The mutagenesis experiment was performed essentially as described by Zoller and Smith [19] using, besides the phosphorylated mutagenic oligonucleotide, a second wild type 5′-OH oligonucleotide primer. Screening of 1000 plaques, obtained after transfection of *E. coli* K58 *ung*$^-$, *sup*$^+$ spheroplasts with ds φX174 DNA synthesized in this way, yielded one mutant phage. DNA sequence analysis of this mutant and wild type φX174 as a control, according to the chain termination method, showed that the ATG start codon of the A* protein has been changed into an ATT codon (fig.1). The mutant will be designated as φX-4499T, indicating the nucleotide change in position 4499 of the φX174 DNA sequence. This nucleotide change alters the

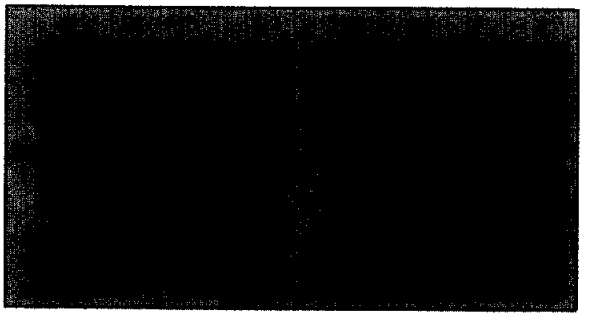

Fig.1. DNA sequence of ϕX-4499T (a) and wild type ϕX174 (b) in the region of the mutation, ϕX no. 4492–4507. The arrow indicates the position of the mutation.

methionine residue in position 173 of the gene A protein into an isoleucine residue. The DNA sequence analysis also revealed that our wild type ϕX174 contains at position 4518 an A residue instead of a G residue as published by Sanger et al. [5] for their *am3cs70* ϕX174 phage. This nucleotide change results in an amino acid substitution of an alanine into a threonine residue in the gene A protein as well as in the A* protein. The ϕX-4499T mutant forms smaller plaques than ϕX174 wild type phage on an *E. coli* C lawn. This smaller plaque morphology is correlated to the burst size of the mutant. The burst size of ϕX-4499T, measured in a one-step growth experiment, is 60 compared to 124 found for wild type ϕX174 in the same experiment.

To analyze if the ϕX-4499T mutant produced A* protein during infection, cell lysates obtained at various times after infection were incubated with the internally ^{32}P-labelled oligonucleotide CAACTTG[^{32}P]ATATTAATAAC. This octadecamer corresponds to the first 18 nucleotides of the ϕX174 replication origin. Upon incubation with gene A protein or A* protein this oligonucleotide is specifically cleaved after the G residue [25]. After cleavage gene A protein and A* protein are radioactive due to covalent linkage of the radioactive oligonucleotide [^{32}P]ATATTAATAAC to a tyrosyl residue of these proteins. The radioactive gene A and A* proteins are separated by polyacrylamide gel electrophoresis. Fig.2 shows the autoradiogram of the polyacrylamide gel of this analysis. As a control the infection was also carried out with wild type ϕX174 and the lysis defective

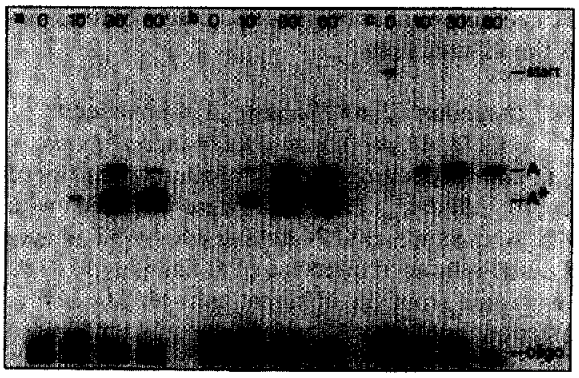

Fig.2. SDS-polyacrylamide gel electrophoresis of cell lysates, obtained at various times after infection of *E. coli* C with wild type ϕX174 (a), ϕX174 *am3* (b) and ϕX-4499T (c), respectively, after reaction with the ^{32}P-labelled octadecamer, CAACTTG[^{32}P]ATATTAATAAC.

ϕX174 *am3*. The infection with ϕX174 *am3* shows increasing amounts of gene A protein and A* protein as the infection proceeds. The drop in the amount of gene A protein (and A* protein) observed 60 min after infection with wild type ϕX174 and ϕX-4499T is due to lysis of the cells. In contrast to wild type ϕX174 and ϕX174 *am3* no A* protein could be detected during infection with the ϕX-4499T mutant. Fig.3 shows a quantitation of

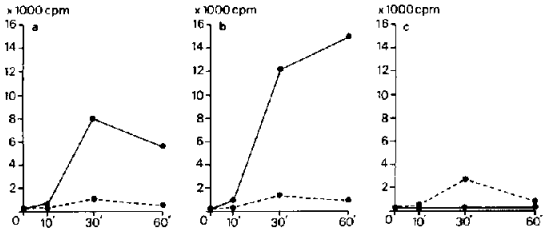

Fig.3. Amount of gene A protein (– – –) and A* protein (——) present at different times after infection of *E. coli* C with ϕX174 wild type (a), ϕX174 *am3* (b), and ϕX-4499T (c), respectively. The relative amounts of gene A protein and A* protein were determined by counting the Čerenkow radiation present in the gel at the position of the gene A protein-oligonucleotide complex and the A* protein-oligonucleotide complex as obtained after reaction with the ^{32}P-labelled octadecamer, CAACTTG[^{32}P]ATATTAATAAC and separation on a SDS-polyacrylamide gel (fig.2).

this result. The level of A* protein in the φX-4499T infected culture is reduced at least 100-fold compared to the wild type infection. Despite the lower burst size of φX-4499T the culture infected with φX-4499T contained even higher levels of the gene A protein than infected wild type cells. It is concluded from these experiments that A* protein, although advantageous for phage reproduction, does not fulfill an essential role in the life cycle of bacteriophage φX174.

4. DISCUSSION

Alteration of the ATG start codon of the A* protein of bacteriophage φX174 into an ATT codon by means of oligonucleotide-directed mutagenesis has resulted in a viable φX174 mutant phage. This mutant φX-4499T, produces functional gene A protein and no A* protein. The 50% lower burst size compared to wild type φX174 is caused either by the changed gene A protein, a methionine → isoleucine substitution in position 173, or by the absence of A* protein. We favor the latter possibility. The shutoff of host DNA replication by the A* protein in wild type infection makes the host replication proteins available for φX174 DNA synthesis. This will increase the number of replicating RF molecules late in infection and may enhance in this way the burst size.

Recently we have proposed a model in which the A* protein is implicated in the switch from stage II (RF→RF) to stage III (RF→ss-DNA-phage) replication [26]. This model is based on the finding that part of the A* molecules carry a covalently bound oligonucleotide, that appeared to arise from cleavage of the viral strand in the intergenic region between the genes J and F [11]. Since free ss viral DNA is not present in the infected cell and since A* protein does not cleave ds φX174 DNA, cleavage of the displaced viral strand of the rolling circle by the A* protein probably takes place. This can only occur if the displaced strand is not packaged. As the A* protein concentration increases during infection, cleavage of the viral strand will block RF→RF replication. No completed circular viral DNA is synthesized and consequently the replication machinery is no longer involved in the synthesis of complementary strand DNA and is available for the synthesis of viral DNA during stage III replication. Recent in vitro studies have shown that the φX174 gene C protein also inhibits viral strand synthesis by binding after a replication round to the replication complex [27]. Addition of proheads results in the starting of stage III replication. So the amount of gene C protein seems to be an important factor in determining the transition of RF replication to ss-DNA synthesis.

Similar results to those here for the A* protein have been obtained for the gene K product of φX174. This gene is also not essential for phage viability, although its absence decreases the burst size 3–6-fold. In this case no clues exist for the profitable function of gene K product during the infection cycle [28].

The initiator protein of the filamentous phages (M13, f_1, f_d), gene II protein, has a similar function to gene A protein during the infection cycle of the isometric phages. The filamentous phage genome also encodes a smaller protein, the gene X protein, which is synthesized from an internal start signal in the same reading frame as the gene II protein. However, site-directed mutagenesis of the ATG start codon of the gene X protein, which was essential for the viability of the filamentous phages [29]. ss-DNA synthesis, but not RF replication was blocked when the gene X protein was absent. Apparently there exists a fundamental difference in the function of the gene X protein of the filamentous phages and the A* protein of the isometric phages. Shutoff of host DNA synthesis, which appeared the most important activity of the A* protein, does not occur in filamentous phage-infected hosts. These cells continue to grow and divide, extruding continuously newly synthesized phage particles.

To our knowledge one example of initiation of prokaryotic protein synthesis using an ATT codon has been described in the literature [30]. The synthesis of the *E. coli* initiation factor IF3 starts effi-

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IF3      : A T T GGAGGAATAAGGTATTAAA
φX-4499T: G C T GGAGGCCTCCACTATTAAA
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Fig.4. Comparison of the DNA sequence of φX-4499T (φX no. 4481–4502) with the DNA sequence at the initiation site of *E. coli* initiation factor IF3. The ATT codon and the GGAGG Shine-Dalgarno sequence are underlined.

ciently on an ATT codon. Comparison of the DNA sequence of ϕX-4999T with the DNA sequence around the initiation site of IF3 synthesis shows a remarkable homology (fig.4). Eight nucleotides upstream from the ATT codon the same, strong Shine-Dalgarno sequence, GGAGG, is found. In both cases the sequence shows a T and an A residue on the 5'- and 3'-sides of the ATT codon respectively. These nucleotides have considerable influence on the formation of the initiation complex probably by extra hydrogen bonds with the nucleotides flanking the anticodon in the anticodon loop of fMet-tRNA [31-34]. Despite these similarities in DNA sequence between the ϕX-4499T mutant and the initiation site of IF3 protein synthesis, no initiation of A* protein synthesis has been observed in ϕX-4499T infected cells. Therefore subtle differences in the primary structure, the sequence between the Shine-Dalgarno sequence and the ATT codon, and the secondary structure may have important consequences for the initiation of protein synthesis.

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REFERENCES

[1] Tessman, E.S. and Tessman, I. (1978) in: The Single-Stranded DNA Phages (Denhardt, D.T. et al. eds) Cold Spring Harbor Monographs Ser., pp. 9-29, Cold Spring Harbor, NY.
[2] Brown, D.R., Hurwitz, J., Reinberg, D. and Zipursky, S.L. (1982) in: The Nucleases (Linn, S.M. and Roberts, R.J., eds) pp. 187-202, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[3] Baas, P.D. (1985) Biochim. Biophys. Acta 825, 111-139.
[4] Langeveld, S.A., Van Mansfeld, A.D.M., Baas, P.D., Jansz, H.S., Van Arkel, G.A. and Weisbeek, P.J. (1978) Nature 265, 417-420.
[5] 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. and Smith, M. (1978) J. Mol. Biol. 125, 225-246.
[6] Van Mansfeld, A.D.M., Van Teeffelen, H.A.A.M., Baas, P.D. and Jansz, H.S. (1986) Nucleic Acids Res. 14, 4229-4238.
[7] Linney, E., Hayashi, M.N. and Hayashi, M. (1972) Virology 50, 381-387.
[8] Van der Ende, A., Langeveld, S.A., Van Arkel, G.A. and Weisbeek, P.J. (1982) Eur. J. Biochem. 124, 245-252.
[9] Langeveld, S.A., Van Mansfeld, A.D.M., Van der Ende, A., Van de Pol, J.H., Van Arkel, G.H. and Weisbeek, P.J. (1981) Nucleic Acids Res. 9, 545-562.
[10] Eisenberg, S. and Finer, M. (1980) Nucleic Acids Res. 8, 5305-5315.
[11] Van Mansfeld, A.D.M., Van Teeffelen, H.A.A.M., Zandberg, J., Baas, P.D., Jansz, H.S., Veeneman, G.H. and Van Boom, J.H. (1982) FEBS Lett. 150, 103-108.
[12] Aoyama, A., Hamatake, R.K. and Hayashi, M. (1983) Proc. Natl. Acad. Sci. USA 80, 4195-4199.
[13] Eisenberg, S. and Ascarelli, R. (1981) Nucleic Acids Res. 9, 1991-2002.
[14] Martin, D.F. and Snover, D. (1976) J. Virol. 18, 141-150.
[15] Funk, F.D. and Snover, D. (1976) J. Virol. 18, 141-150.
[16] Colasanti, J. and Denhardt, D.T. (1985) J. Virol. 53, 807-813.
[17] Baas, P.D., Van Teeffelen, H.A.A.M., Teertstra, W.R., Jansz, H.S., Veeneman, G.H., Van der Marel, G.A. and Van Boom, J.H. (1980) FEBS Lett. 110, 15-20.
[18] Hutchison, C.A. iii and Sinsheimer, R.L. (1966) J. Mol. Biol. 18, 429-447.
[19] Zoller, M.J. and Smith, M. (1984) DNA 3, 479-488.
[20] Wallace, R.B., Schaffer, J., Murphy, R.F., Bonner, J., Hirose, T. and Itakura, K. (1979) Nucleic Acids Res. 6, 3543-3557.
[21] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463-5467.
[22] Sanger, F., Coulson, A.R., Barrell, B.G., Smith, A.J.H. and Roe, B.A. (1980) J. Mol. Biol. 143, 161-178.
[23] Laemmli, U.K. (1970) Nature 227, 680-685.
[24] Kozak, M. (1983) Microbiol. Rev. 1-45.
[25] Van Mansfeld, A.D.M., Van Teeffelen, H.A.A.M., Baas, P.D., Veeneman, G.H., Van Boom, J.H. and Jansz, H.S. (1984) FEBS Lett. 173, 351-356.
[26] Van Mansfeld, A.D.M., Van Teeffelen, H.A.A.M., Fluit, A.C., Baas, P.D. and Jansz, H.S. (1986) Nucleic Acids Res. 14, 1845-1861.
[27] Aoyama, A. and Hayashi, M. (1986) Cell 47, 99-106.

[28] Gillam, S., Atkinson, T., Markam, A. and Smith, M. (1985) J. Virol. 53, 708–709.
[29] Fulford, W. and Model, P. (1984) J. Mol. Biol. 178, 137–153.
[30] Sacerdot, C., Fayat, G., Dessen, P., Springer, M., Plumbridge, J.A., Grunberg-Manago, M. and Blanquet, S. (1982) EMBO J. 1, 311–315.
[31] Taniguchi, T. and Weissmann, C. (1978) J. Mol. Biol. 118, 533–565.
[32] Belin, D., Hedgpeth, J., Selzer, G.B. and Epstein, R.H. (1979) Proc. Natl. Acad. Sci. USA 76, 700–704.
[33] Ganoza, M.C., Frazer, A.R. and Neilson, T. (1978) Biochemistry 17, 2769–2775.
[34] Steitz, J.A., Sprague, K.V., Steege, D.A., Yuan, R.C., Laughrea, M., Moore, P.B. and Wahba, A.J. (1977) in: Nucleic Acid Protein Recognition (Vogel, H.J. ed.) pp. 491–508, Academic Press, New York.