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The antirepressor of phage P1

Isolation and interaction with the C1 repressor of P1 and P7

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Two antirepressor proteins, Ant1 and Ant2, of molecular weight 42 and 32 kDa, respectively, are encoded by P1 as a single open reading frame, with the smaller protein initiating at an in-frame start codon. Another open reading frame, *icd*, 5′ upstream of and overlapping *ant*1 is required for *ant*1 expression. Using appropriate *ant* gene-carrying plasmids we have overproduced and purified Ant1/2 in the form of a protein complex and Ant2 as a single protein. Sequence analysis confirmed the N-terminal amino acids predicted from the DNA sequence of *ant1*/*ant2*, except that the N-terminal methionine is missing in the Ant2 protein. Under appropriate conditions the C1 repressors of phages P1 and P7 specifically co-precipitate with the Ant1/2 complex but not with Ant2 protein alone. The results suggest that the antirepressor may exert its C1-inactivating function by a direct protein–protein interaction.

Regulatory protein; Translational coupling; In-frame start codon; T7 RNA polymerase transcription/translation system

1. INTRODUCTION

P1 and P7 are closely related temperate phages which have a similar genome size (~100 kb) and organization [1]. Both phages encode (i) a primary repressor protein, C1, which represses the lytic functions and (ii) a secondary repressor, C4, which is an antisense RNA [2]: it represses the synthesis of an antirepressor, Ant, that would otherwise inactivate the C1 repressor [3]. Nevertheless, P1 and P7 are reciprocally heteroimmune, i.e. each will grow on the lysogen of the other [4,5]. This immunity difference has been localized by genetic means to the *c*4 region [4]. The *c*1 genes of P1 and P7 can be exchanged without altering the immunity specificity, i.e. both C1 repressor proteins must be functionally identical [4,5]. In spite of this identity P1 or P7 virulent mutants that constitutively synthesize Ant only induce the homoimmune but not the heteroimmune resident prophage upon infection [5,6]. These results indicated that the antirepressor is not capable of recognizing and inactivating the C1 repressor directly. Further experiments suggested the existence of a phage-specific loading site, called *sas* (for site of ant specificity), at which only the homologous antirepressor acts [3,7]. The loading site hypothesis, although helpful in explaining the indirect action of Ant, does not shed light on the mechanism by which C1 repressor is inactivated. Therefore, a direct interaction of Ant and C1 appears to be necessary once the antirepressor is channeled via the phage-specific *sas* site.

In a first approach to study the function(s) of P1 antirepressor we have investigated its interaction with the P1 and P7 repressors in vitro. By using appropriate P1 DNA containing recombinant plasmids the antirepressor of P1 and the C1 repressors of P1 and P7 have been overproduced. We describe the purification of the P1 antirepressor which consists of two proteins, Ant1 and Ant2. We show that purified P1 antirepressor is capable of specifically co-precipitating the C1 repressor of P1 and P7 from cellular crude extracts, indicating a direct repressor–antirepressor interaction.

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Abbreviations: kb, kilobase; bp, base pairs; kDa, kilodalton; IPTG, isopropyl-β-D-thiogalactopyranoside; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

2. MATERIALS AND METHODS

2.1. *Reagents*

Sources were as follows: IPTG, Boehringer-Mannheim; guanidine-HCl, Sigma; hydroxylapatite and low molecular weight protein standards, Bio-Rad. Other reagents have been described previously [8–10]. Buffer A: 20 mM Tris-HCl, pH 7.6, 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerol. Buffer B: 20 mM Tris-phosphate, pH 8.0, 50 mM KH_2PO_4, 3 M guanidine-HCl, 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, SDS buffer: 90 mM Tris-HCl, pH 6.8, 5.7% SDS, 7% 2-mercaptoethanol, 12.5% glycerol.

2.2. *Bacteria, phage and plasmids*

The *Escherichia coli* strains (with relevant genotypes) used were JM83F′ sup° strep^r, tet^r [11,12] and C600 *sup*E. The recombinant phage used was mGP1-2, containing the T7 RNA polymerase gene 1 inserted into M12mp18 under the control of the *lac* promoter ([13]; Tabor and Richardson, personal communication). Plasmids pAH1018 and pAH1016 contain different parts of the P1 *imm*I DNA (Fig. 1) inserted into plasmid pT7-6 with the T7 RNA polymerase promoter

ϕ10 [11,13]. Plasmid pMV2w carries the *c*1 gene on a 1.634 kb $MscI_{693}$–$BclI_{2327}$ subfragment of P1 *Eco*RI fragment 7 (6.0 kb) inserted into the vector, pJF118EH (*tac*) [8,14]. Plasmid pMV2w7 carries the *c*1 gene of P7 on a 1.45 kb $MscI_{693}$–$AvaI_?$ subfragment inserted into the same vector. In isolating this subfragment from a P7 $BamHI_{641}$– $AvaI_?$ fragment inserted into pBR322 (a plasmid provided by F. Osborne), 52 bp upstream of the *c*1 gene were deleted. Index numbers refer to the distance (in bp) from the P1 or P7 loxP site [1]. A question mark indicates that the nucleotide position is not yet known.

2.3. *Preparation of C1 repressor and antirepressor*

The C1 repressor proteins of P1 and P7 were overproduced upon induction by IPTG of strain C600/pMV2w and C600/pMV2w7, respectively, and were purified as described [8]. P1 antirepressor was overproduced by infection of strain JM83F′/pAH1018 (*ant*1/2) or JM83F′/pAH1016 (*ant*2) with phage mGP1-2 in the presence of IPTG [11]. The purified antirepressor proteins, Ant1/2 and Ant2, were kept at −20°C in buffer B and A containing 1.5 M NaCl, respectively.

2.4. *N-Terminal amino acid analysis*

The antirepressor proteins, Ant1 and Ant2, were separated by 15% SDS-PAGE and blotted onto a polyvinylidine difluoride membrane as described [15]. The polypeptides were sequenced in a pulse-liquid phase sequencer, model 477A (Applied Biosystems), equipped with the model 120 PTH amino acid analyser as described [16].

3. RESULTS

3.1. *Overproduction of P1 antirepressor*

As a consequence of antirepressor synthesis from a multicopy plasmid the bacteria become filamentous and finally die [11]. Therefore we have used the pT7-6/mGP1-2 expression system of Tabor and Richardson [13] for the overproduction of P1 antirepressor. Bacteria carrying appropriate pT7-6/P1 *imm*I recombinant plasmids were infected with the recombinant phage mGP1-2, and synthesis of T7 RNA polymerase was induced by IPTG as described in the legend to Fig. 2. As soon as the enzyme is synthesized, transcription starts at the T7 RNA polymerase promoter ϕ10 located on the pT7-6/P1 *imm*I plasmid (Fig. 1). Plasmid pAH1018 contains the intact *icd* and *ant* genes, which are translationally coupled, and part of the *kil*A gene [17,18]. In plasmid pAH1016, *icd* and the beginning of the *ant*1 gene are deleted so that only *ant*2 can be expressed (Fig. 1). Following a 2 h incubation period at 37°C the bacteria were lysed by either SDS or lysozyme as described in the legend to Fig. 2 and the lysates subjected to SDS-PAGE. Under these conditions the Ant1/2 proteins and a KilA* fusion protein [11] are overproduced from pAH1018 (Fig. 2A), whereas pAH1016 yields only Ant2 protein (Fig. 2B).

3.2. *Purification of P1 antirepressor*

Following the overproduction of the Ant1/2 proteins in strain JM83F′/pAH1018 the bacteria were lysed with lysozyme as described in the legend to Fig. 2. An aliquot of the lysozyme lysate was subjected to SDS-PAGE. SDS lysates of the IPTG-induced and uninduced cultures were run in parallel in order to follow the overproduction of SDS-soluble antirepressor proteins. Very little or no Ant1/2 proteins were solubilized by the lysozyme procedure (Fraction S, Fig. 2A). However, when the remaining pellet was extracted with buffer A

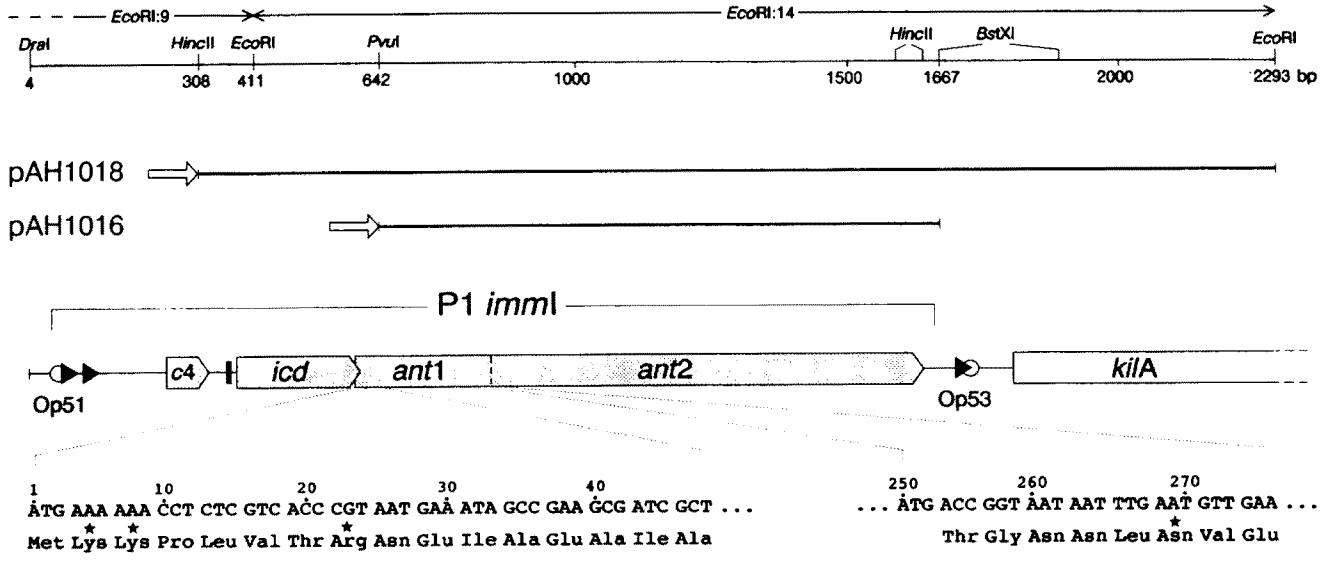

Fig. 1. The P1 *imm*I operon and the P1 *imm*I/pT7-6 recombinant plasmids used. P1 *imm*I DNA overlaps the neighbouring P1 *Eco*RI fragments 9 and 14, and contains the C1-controlled operator Op51 (open circle), the tandem promoter P51a and b (black triangles), and the genes, *c*4, *icd*, and *ant*, in that order. It is followed by the C1-controlled Op53/P53 element and the *kil*A gene which extends from P1 *Eco*RI:14 into the neighbouring P1 *Eco*RI:25 (not shown). The *icd* gene comprises 73 codons and is preceded by a ribosome binding site (black rectangle). The *ant*1 gene codes for 347 amino acids and contains an in-frame start (vertical interrupted line) for a polypeptide, Ant2, of 264 amino acids. The *icd* and *ant*1 genes overlap and are translationally coupled. The nucleotide triplets at the 5′ ends of *ant*1 and *ant*2 are shown together with the amino acids which were determined as described in section 2. A star means that the amino acid was not identified unequivocally. Plasmid pAH1018 and pAH1016 contains the P1 *Hin*cII–*Eco*RI and P1 *Pvu*I–*Bst*XI subfragments, respectively (horizontal line) inserted into pT7-6 with the T7 ϕ10 promoter (bold arrow). Relevant restriction enzyme cleavage sites with the nucleotide positions are shown.

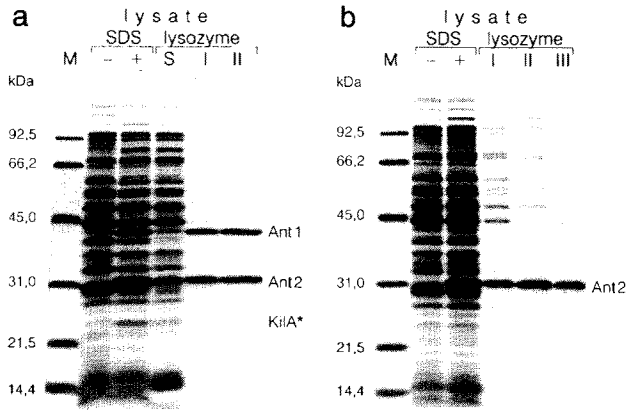

Fig. 2. SDS-PAGE of overproduced and purified Ant1/Ant2 proteins. *Lysozyme lysate*: A 1 l culture of strain JM83F′ carrying either plasmid pAH1018 (a) or pAH1016 (b) was grown at 37°C. At an OD_{590} = 1.0, cells were infected with phage mGP1-2 (multiplicity of infection = 20) and synthesis of T7 RNA polymerase was induced by the addition of IPTG (2 mM final concentration). After 2 h bacteria were centrifuged (5 min at 8,000 × *g*, 2°C, GSA rotor) and the cell pellet was resuspended in 200 mM NaCl, 20 mM spermidine, and 2.5 mM EDTA (5 ml/g wet cell paste). All operations were done at 0–4°C if not otherwise noted. The suspension was adjusted to 25 mM Tris-HCl, pH 8.0, 180 mM NaCl, 2 mM EDTA, 15 mM spermidine, 3.5% sucrose, 0.1% Brij 58, 0.5 mg/ml lysozyme. After 30 min incubation lysis was completed by warming up to 10°C for 3 min. (a) The lysozyme lysate (20 ml) was adjusted to 1.5 M NaCl and centrifuged (10 min at 20,000 × *g*, SS34 rotor). The supernatant (S) was removed and the pellet was washed with 10 ml of buffer A containing 1.5 M NaCl and centrifuged. It was extracted with 10 ml of buffer A containing 3 M guanidine-HCl for 10 min and again centrifuged. The supernatant contained the Ant1/2 proteins (Fraction I). Fraction I was dialyzed overnight against buffer A containing 6 M urea. The Ant1/2 proteins precipitated under these conditions. The precipitate was incubated in 5 ml buffer A with 5 mM $MgCl_2$, DNase I and RNase I (50 μg/ml each) for 5 min at 30°C. After extensive washing with buffer A the precipitate was dissolved in buffer B and applied to a hydroxylapatite column (2 ml, equilibrated with buffer B) and the flow-through was collected (Fraction II). (b) The lysozyme lysate (20 ml) was centrifuged (10 min at 20,000 × *g*, SS34 rotor), the pellet was eluted with 10 ml of buffer A containing 1.5 M NaCl and then again centrifuged. More than 60% of the total Ant2 protein was found in the supernatant (Fraction I). Solid ammonium sulfate (0.242 g/ml) was slowly added to Fraction I. After 2 h the precipitate was collected by centrifugation (10 min at 12,000 × *g*, SS34 rotor) and dissolved in buffer A with 1.5 M NaCl (Fraction II). Fraction II was dialyzed overnight against buffer A containing 50 mM NaCl. The precipitate was washed with the same buffer and then dissolved in 2 ml buffer A containing 1.5 M NaCl (Fraction III). *SDS lysate:* A 10 ml culture of uninduced (−) and a 10 ml aliquot of the IPTG-induced (+) bacteria were separately centrifuged. Lysates of the cell pellets were prepared in SDS (1 ml bacterial culture, OD_{450} = 1, 30 μl SDS buffer, 5–10 min at 100°C). Aliquots of the fractions were subjected to 15% SDS-PAGE and the proteins were stained with Coomassie blue. M, marker proteins, in descending order: phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme.

containing 3 M guanidine-HCl both proteins were solubilized in about equimolar amounts (Fraction I, Fig. 2A). Fraction I was treated with DNase and RNase in order to remove contaminating nucleic acids and passed through a hydroxylapatite column. The Ant1/2 proteins appear in the flow-through and are at least 90% pure (Fraction II, Fig. 2A). Since both proteins co-purify under all conditions tested, strain JM83F′/pAH1016 was used for the isolation of Ant2 protein alone. In contrast to the mixture of Ant1 and Ant2, the Ant2 protein is already solubilized by extraction of the cell pellet with buffer A containing 1.5 M NaCl (Fraction I, Fig. 2B) and only precipitates at salt concentrations of less than 0.5 M NaCl. The protein was further purified by precipitation with ammonium sulfate (Fraction II, Fig. 2B) and by dialysis against buffer A containing 50 mM NaCl (Fraction III, Fig. 2B) as described in the legend to Fig. 2. Fraction III contains the Ant2 protein at least 80% pure. The different solubility properties of Ant1/2 vs. the Ant2 protein indicate that the former proteins exist as a complex containing about equimolar amounts of Ant1 and Ant2. For convenience, in the following we call this complex the antirepressor.

The N-terminal amino acids of the Ant1 and Ant2 protein were determined as described in section 2 using 100 μg of antirepressor protein (Fraction II). Most of the N-terminal amino acids shown in Fig. 1 could be identified unequivocally, and their sequence corresponds to that deduced from the nucleotide sequence of the *ant*1/2 gene [11]. The Ant2 protein does not contain an N-terminal methionine (Fig. 1), so that the molecule contains a total of 263 instead of 264 amino acids.

3.3. *Interaction of P1 antirepressor with the C1 repressor of P1 and P7*

Purified P1 C1 repressor samples (2 μg each in 200 μl of buffer A) were mixed with increasing amounts of

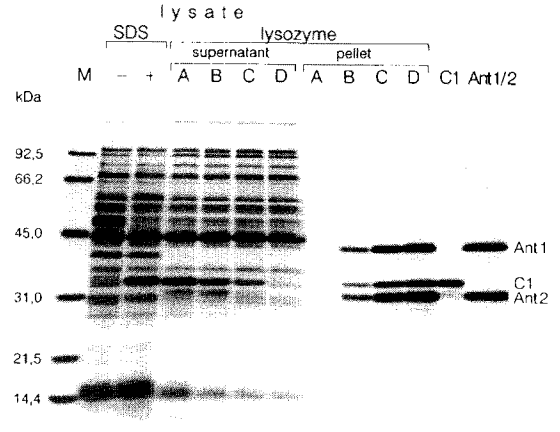

Fig. 3. Precipitation of P1 C1 repressor by antirepressor. A lysozyme lysate of an IPTG-induced culture of C600/pMV2w was diluted 10-fold with buffer A. Samples (200 μl each) were incubated with 0 (A), 5 (B), 10 (C), and 20 μg (D) of antirepressor for 30 min at 37°C. The samples were centrifuged (10 min at 20,000 × *g*, 2°C, SS34 rotor) and the supernatants carefully removed. The pellets were washed once with 0.5 ml of buffer A and then redissolved in 50 μl of SDS buffer. Supernatants and redissolved pellets (50 and 12.5 μl each, respectively) were subjected to 15% SDS-PAGE. For the preparation of the lysozyme and SDS lysates of uninduced (−) and induced (+) bacteria, electrophoresis, marker proteins, and protein staining see the legend to Fig. 2. M, marker proteins; C1, 2.5 μg of P1 C1 repressor; Ant1/2, 10 μg of antirepressor (Fraction II).

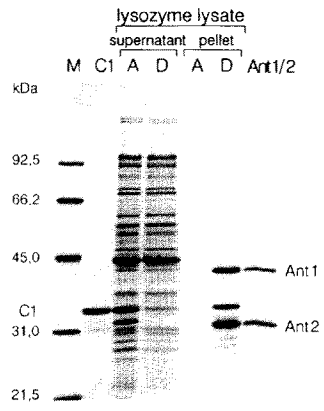

Fig. 4. Precipitation of P7 C1 repressor by P1 antirepressor. A lysozyme lysate of an IPTG-induced culture of C600/pMV2w7 was treated in the same way as described in the legend to Fig. 3 except that 0 (A) and 20 μg (D) of P1 antirepressor (Fraction II) was used. For marker proteins, electrophoresis and protein staining see the legend to Fig. 2. M, marker proteins; C1, 2.5 μg of P7 C1 repressor; Ant1/2, 5 μg of antirepressor (Fraction II).

BSA (2–100 μg) and subsequently incubated with freshly diluted antirepressor (8 μg each of Fraction II). The antirepressor precipitated under these conditions and only C1 repressor, but not BSA, was found in the redissolved precipitate (data not shown). The specificity of the repressor–antirepressor interaction was best demonstrated in the following experiment. Increasing amounts of antirepressor (Fraction II, A–D) were added to a lysozyme lysate of C600/pMV2w in which P1 C1 repressor had been overproduced by induction with IPTG (Fig. 3). A precipitate was pelleted and both the supernatant and the redissolved pellet were subjected to SDS-PAGE. SDS lysates of the IPTG-induced and uninduced cultures were run in parallel in order to follow the overproduction of C1 repressor. The C1 repressor band reappeared in the pellet in the same measure as it disappeared from the supernatant. As judged from the intensity of the Coomassie blue-stained protein bands, a 2- to 4-fold molar excess of antirepressor is needed to precipitate a given amount of C1 repressor (Fig. 3). The reaction is specific for the Ant1/2 protein complex, since Ant2 protein (Fraction III), when mixed with purified C1 repressor in a 4:1 weight ratio, precipitates, but the precipitate does not contain C1 repressor (data not shown).

We tested whether the C1 repressor of P7 would also react with the P1 antirepressor. Following induction of C600/pMV2w7 with IPTG a 34 kDa protein is overproduced which was considered to be the P7 C1 repressor. To prove this assumption we have purified this protein following exactly the procedure described for the purification of the P1 C1 repressor [8]. The purified P7 C1 repressor (Fig. 4) specifically binds to operator-containing P1 DNA (data not shown). A lysozyme lysate of induced C600/pMV2w7 bacteria was incubated with antirepressor as described above. The 34 kDa protein is precipitated from the lysate with antirepressor (Fig. 4) as was shown above for the P1 C1 repressor.

4. DISCUSSION

Sequence analysis confirmed the N-terminal amino acids predicted from the DNA sequence of *ant*1 [11]. The Ant2 protein lacks only the N-terminal methionine. The analysis also confirms the existence of an overlap of the termination-initiation sites, ATGA (nucleotides 1–4, Fig. 1), of the *icd ant*1 genes [17]. Such an overlap indicates a translational coupling of the genes and is frequently found for phage genes [19].

The C1 repressors of P1 and P7 are not only functionally identical but also very similar in their structure. The *c*1 regions of both phages contain open reading frames of 283 codons each, differing only by two amino acids [20]. Therefore, it is not surprising that the C1 repressor of P7 also co-precipitates with the antirepressor of P1. The extensive similarity of both repressors strongly supports the idea that the inducibility by the antirepressor of only the homoimmune prophage must reside in some phage-specific element other than C1 [3]. This element may be the hypothetical DNA site, *sas* [3,7], which mediates an interaction with the antirepressor of the same phage only (or preferentially). A phage-specific interaction of the antirepressor with a *sas* site requires that Ant1/2 of P1 and P7 differ from each other but without affecting their ability to interact with the C1 repressor of either phage.

The properties of P1 antirepressor are similar to those of the antirepressor of phage P22. The latter interferes directly with the C1 repressor of phage λ which is analogous in function to the C2 repressor of P22 [21]. Preincubation of λ C1 repressor with P22 antirepressor in P22-infected crude cell extracts abolishes the subsequent binding of λ C1 repressor to λ DNA [22]. The 35-kDa antirepressor of P22 is also solubilized in guanidine-HCl by extracting the insoluble material from a lysate of P22-infected bacteria [23].

Precipitation with P1 antirepressor of the P1 or P7 repressor from cellular crude extracts is a remarkably specific reaction. Nevertheless, we are aware of the fact that it does not mimic the in vivo conditions simply because of the solubility properties of the P1 antirepressor. To overcome this problem we are currently trying to isolate fragments of the P1 antirepressor protein in the hope that they may retain the ability to interact with the C1 repressors under non-denaturing conditions.

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REFERENCES

[1] Yarmolinsky, M.B. and Lobocka, M.B. (1993) in: Genetic Maps, Book 1 (O'Brien, S.J. ed.) pp. 1.50–1.61, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[2] Citron, M. and Schuster, H. (1990) Cell 62, 591–598.
[3] Yarmolinsky, M.B. and Sternberg, N. (1988) in: The Bacteriophages, vol. 1 (Calendar, R. ed.) pp. 291–438, Plenum, NY.
[4] Chesney, R.H. and Scott, J.R. (1975) Virology 67, 375–384.
[5] Wandersman, C. and Yarmolinsky, M. (1977) Virology 77, 386–400.
[6] West, B.W. and Scott, J.R. (1977) Virology 78, 267–276.
[7] Sternberg, N., Austin, S. and Yarmolinsky, M. (1979) Contrib. Microbiol. Immunol. 6, 89–99.
[8] Dreiseikelmann, B., Velleman, M. and Schuster, H. (1988) J. Biol. Chem. 263, 1391–1397.
[9] Heinrich, J., Riedel, H.-D., Baumstark, B.R., Kimura, M. and Schuster, H. (1989) Nucleic Acids Res. 17, 7681–7692.
[10] Velleman, M., Dreiseikelmann, B. and Schuster, H. (1987) Proc. Natl. Acad. Sci. USA 84, 5570–5574.
[11] Heisig, A., Riedel, H.-D., Dobrinski, B., Lurz, R. and Schuster, H. (1989) J. Mol. Biol. 209, 525–538.
[12] Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) Gene 33, 103–119.
[13] Tabor, S. and Richardson, C.C. (1985) Proc. Natl. Acad. Sci. USA 82, 1074–1078.
[14] Fürste, J.P., Pansegrau, W., Frank, R., Blöcker, H., Scholz, P., Bagdasarian, M. and Lanka, E. (1986) Gene 48, 119–131.
[15] Choli, T., Kapp, U. and Wittmann-Liebold, B. (1989) J. Chromatogr. 476, 59–72.
[16] Walsh, M., McDougall, J. and Wittmann-Liebold, B. (1988) Biochemistry 27, 6867–6876.
[17] Riedel, H.-D., Heinrich, J. and Schuster, H. (1993) J. Bacteriol. 175, 2833–2838.
[18] Hansen, E.B. (1989) J. Mol. Biol. 207, 135–149.
[19] Sanger, F., Coulson, A.R., Hong, G.F., Hill, D.F. and Peterson, G.B. (1982) J. Mol. Biol. 162, 729–773.
[20] Osborne, F.A., Stovall, S.R. and Baumstark, B.R. (1989) Nucleic Acids Res. 17, 7671–7680.
[21] Susskind, M.M. and Youderian, P. (1983) in: Lambda II (Hendrix, R.W., Roberts, J.W., Stahl, F.W. and Weisberg, R.A., eds) pp. 347–363, Cold Spring Harbor Laboratory, Cold Spring Habor, NY.
[22] Sauer, R.T., Krovatin, W., DeAnda, J., Youderian, P. and Susskind, M.M. (1983) J. Mol. Biol. 168, 699–713.
[23] Susskind, M.M. and Botstein, D. (1975) J. Mol. Biol. 98, 413–424.