SYNTHESIS OF A POLYNUCLEOTIDE CORRESPONDING TO THE PROMOTER REGION OF BACTERIOPHAGE fd DNA

Yu. A. OVCHINNIKOV, V. A. EFIMOV and O. G. CHAKHMAKCHEVA
Shemyakin Institute of Bioorganic Chemistry, USSR Academy of Sciences, Moscow, 117988, USSR

Received 22 February 1979

1. Introduction

The interaction of RNA polymerase with the promoter regions of DNA during initiation of the transcription process is a highly interesting example of nucleic acid–protein recognition. Structure–functional study of the components of this interaction has now become a reality [1–6] owing to elucidation of the nucleotide sequence of a number of bacterial and viral promoters [1] and the investigations to establish the primary structure of *Escherichia coli* RNA polymerase [7].

The success of such a correlation depends largely on the availability of adequate amounts of both components in a sufficiently pure state. A highly promising approach to obtain a promoter region is its chemical–enzymatic synthesis [5]. To apply this approach one must be able to systematically modify the various parts of the DNA promoter, whether by deletion, substitution of individual base pairs (or even of large fragments) or insertion of modified nucleotide analogs, which among other things would permit pinpointing the sites of attachment of RNA polymerase to the nucleic acid molecule.

In the process of studying the functional topography of *E. coli* RNA polymerase [6], we have carried out the chemical–enzymatic synthesis of an 86-nucleotide-long duplex DNA representing the promoter region G2 of phage fd DNA [8] and containing 15 base pairs in the postinitiation region and 71 pairs in the promoter region (see [9]).

The first major consideration in favor of this fragment was the fact that it was one of the strongest promoter regions, the structure of which had been determined by two independent groups at the time this work was started [8,10]. A second consideration was the ready accessibility of the circular single-stranded form of the native fd DNA. This fact facilitated the development of the two new chemical–enzymatic methods for synthesis of bihelical DNA described here using the example of the G2 promoter of bacteriophage fd DNA.

2. Materials and methods

Deoxynucleoside triphosphates were purchased from Calbiochem; [γ-^{32}P]ATP (20 Ci/mmol and 3000 Ci/mmol) from Amersham; bacterial alkaline phosphatase, snake venom phosphodiesterase (VPDE), spleen phosphodiesterase (SPDE) and DNase I from Worthington; micrococcal deoxyribonuclease and S_1 nuclease from P-L Biochemicals. T_4 polynucleotide kinase, T_4 polynucleotide ligase and T_4 DNA polymerase were prepared according to [11].

Purified phage fd and single-stranded DNA (SS DNA) were prepared by the method in [12], except that the phage was purified by precipitation with polyethyleneglycol (6000) [13].

Electrophoresis was carried out on vertical slabs of polyacrylamide gel using 0.05 M Tris–borate (pH 8.3)/1 mM EDTA as electrode buffer.

Sequence determination of the synthetic polynucleotides was performed by chemical means, inserting a ^{32}P-label in the 5′-terminus, separating the chains, and determining the positions of the pyrimidines and of guanines as in [14]; the purine (A+G) positions were found by acidic apurinization [15,16].

For the chemical synthesis of the oligodeoxyribonucleotides use was made of the phosphodiester method [17]. The synthetic intermediates were characterized by ultraviolet spectra and paper or thin-layer chromatography, and their monomer composition was determined by enzymatic digestion in the presence of snake venom phosphodiesterase. The nucleotide sequence of the final segments (A–H), after insertion of the 5′-^{32}P-label [18], was confirmed by the two-dimensional techniques [19].

A typical reaction mixture for the T_4 polynucleotide ligase catalyzed joining of 5′-^{32}P-oligonucleotides contained the following components: 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 0.6 mM ATP, 1.3 μM phage fd DNA [(+)-strand], 4–8 μM of each oligonucleotide and 48 units enzyme/100 μl. Before adding the ATP, DTT and ligase the mixture was heated at 90°C for 3 min, then slowly cooled to room temperature. The reaction was carried out at 20°C for 2–4 h and terminated by adding EDTA to 0.05 M. The course of the reaction was followed by measuring the increase in the [^{32}P]phosphate fraction resistant to alkaline phosphatase [20] and by electrophoresis in 10% polyacrylamide gel.

A typical incubation mixture for polynucleotide synthesis by the limited template copying method contained 0.02 M Tris–HCl (pH 7.6–8.0), 0.1 mM $MgCl_2$, 0.5 mM DTT, 0.05–0.20 M KCl, 0.33 mM of each deoxyribonucleoside triphosphate, 0.25 μM fd SS DNA, 1.26 μM of primer and 1.25–10 μM 'stopper' together with 30 units T_4 DNA polymerase/100 μl.

The cleavage of the single-stranded regions by S_1 nuclease was carried out in a 0.03 M sodium acetate buffer solution (pH 5.0) containing 0.3 M NaCl, 1 mM $ZnCl_2$ and 0.5 μM of the promoter minus strand–SS fd DNA complex. After adding 10 μg phage fd DNA and 8 units S_1 nuclease/100 μl the mixture was allowed to stand at 20°C for 1 h. The reaction was terminated by adding EDTA to 0.05 M. The duplexes were isolated by gel filtration on a Bio-Gel A-1.5 m column (1 × 50 cm) using 0.1 M triethylammonium bicarbonate at 4°C.

3. Results and discussion

The methods devised here for synthesis of bihelical DNA fragments were based on the inherent ability of oligo- and polynucleotide strands, owing to base pairing, to form ordered double-stranded complexes with their structurally complementary regions of the single-stranded DNA. Both these methods suppose that only one strand of the promoter region had to be synthesized, the second strand of the duplex being taken from the corresponding region of the native phage fd DNA.

That synthetic oligonucleotides can be joined together on natural single-stranded templates had been shown earlier [21]. Our first method issued from these experiments and involved the covalent binding of the synthetic segments, in their sum total comprising the minus strand of the promoter, on the single-stranded fd phage DNA, as on a template, in the presence of DNA ligase, followed by cutting out the resultant duplex (see fig.1).

To achieve this the sequence of promoter minus strand was divided into eight segments (A–H), each 10–13 nucleotides in length, which were prepared by chemical means. The synthesized oligonucleotides were then 5′-phosphorylated using [γ-^{32}P]ATP and

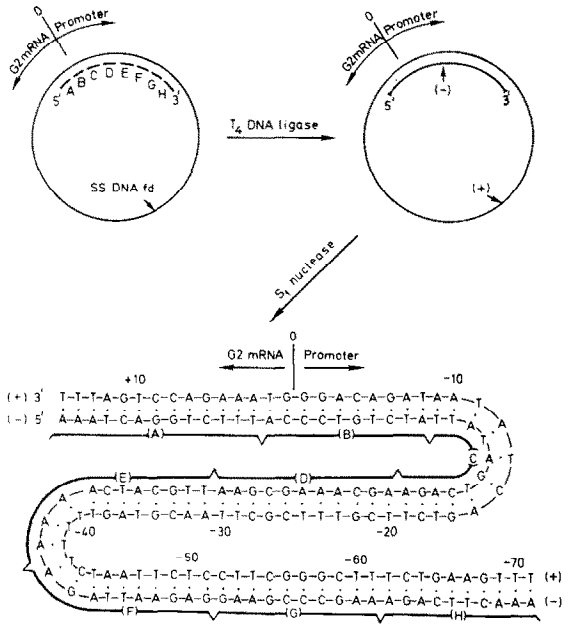

Fig.1. Plan for the synthesis of the G2 promoter region of fd phage DNA by the first method, and the nucleotide sequence of this promoter. (A–H) - oligonucleotides.

T_4 polynucleotide kinase [18], hybridized with plus strand DNA from wild-type fd phage preliminarily isolated in the individual state and then joined together using the T_4 polynucleotide ligase. This gave the minus strand of the promoter. As second strand use was made of the SS fd DNA region, corresponding to the plus strand of the promoter. Thus the desired duplex was obtained by treatment of the synthetic minus strand–circular DNA complex with single-strand-specific S_1 nuclease [22].

In a similar way the synthesis of four shortened variants of the same DNA promoter region: namely 45 nucleotides [duplex (A–D)], 56 nucleotides [duplex (A–E)], 66 nucleotides [duplex (A–F)] and 76 nucleotides [duplex (A–G)] long, each containing 15 base pairs in the postinitiation region and 30, 41, 51 and 61 pairs, respectively, into the promoter region was carried out. The correctness of the joining of the synthetic segments was demonstrated (after dephosphorylation of the duplexes with bacterial alkaline phosphatase) by degradation to 5′- and 3′-nucleotides (nearest neighbor analysis, see table 1) [20], whereas the homogeneity of the duplexes was proved by electrophoresis in 8% non-denaturating polyacrylamide gel (fig.2). The structure of the 86-nucleotide-long fragment (A–H) was confirmed by a

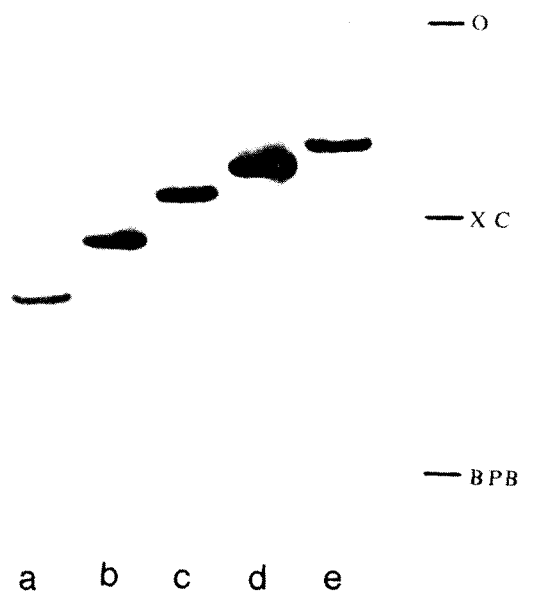

Fig.2. Electrophoresis of synthetic duplexes on polyacrylamide slab gel (20 × 20 × 0.15 cm) using 0.05 M Tris–borate (pH 8.3)/1 mM EDTA. (a), duplex (A–D); (b), duplex (A–E); (c), duplex (A–F); (d), duplex (A–G) and (e), duplex (A–H). O, origin; XC, xylene cyanol dye; BPB, bromophenol blue dye.

Table 1
Phosphodiesterase degradation of synthetic duplexes[a]

Duplex analyzed[b]	^{32}P Radioactivity (cpm)							
	5′-Mononucleotide analysis (VPDE)				3′-Mononucleotide analysis (SPDE)			
	dpT	dpC	dpA	dpG	dTp	dCp	dAp	dGp
(A–D)	8300 (1.0)	<100	15 900 (1.91)	<100	2500 (1.0)	<100	5100 (2.04)	<100
(A–E)	12 400 (2.0)	<100	13 100 (2.11)	<100	3300 (1.0)	<100	9800 (2.97)	<100
(A–F)	14 200 (2.03)	<100	21 000 (3.0)	<100	4200 (1.0)	<100	15 900 (3.79)	<100
(A–G)	10 500 (2.1)	<100	14 800 (2.96)	5000 (1.0)	3700 (1.03)	<100	13 500 (3.75)	3600 (1.0)
(A–H)	7900 (2.02)	<100	16 200 (4.15)	3900 (1.0)[b]	2200 (1.0)	<100	11 200 (5.09)	2300 (1.04)

[a] After removal of the 5′-end phosphate by the action of *E. coli* alkaline phosphatase
[b] See fig.1

Values in parentheses represent the molar ratio as determined from specific activity

Abbreviations: VPDE, snake venom phosphodiesterase; SPDE, spleen phosphodiesterase

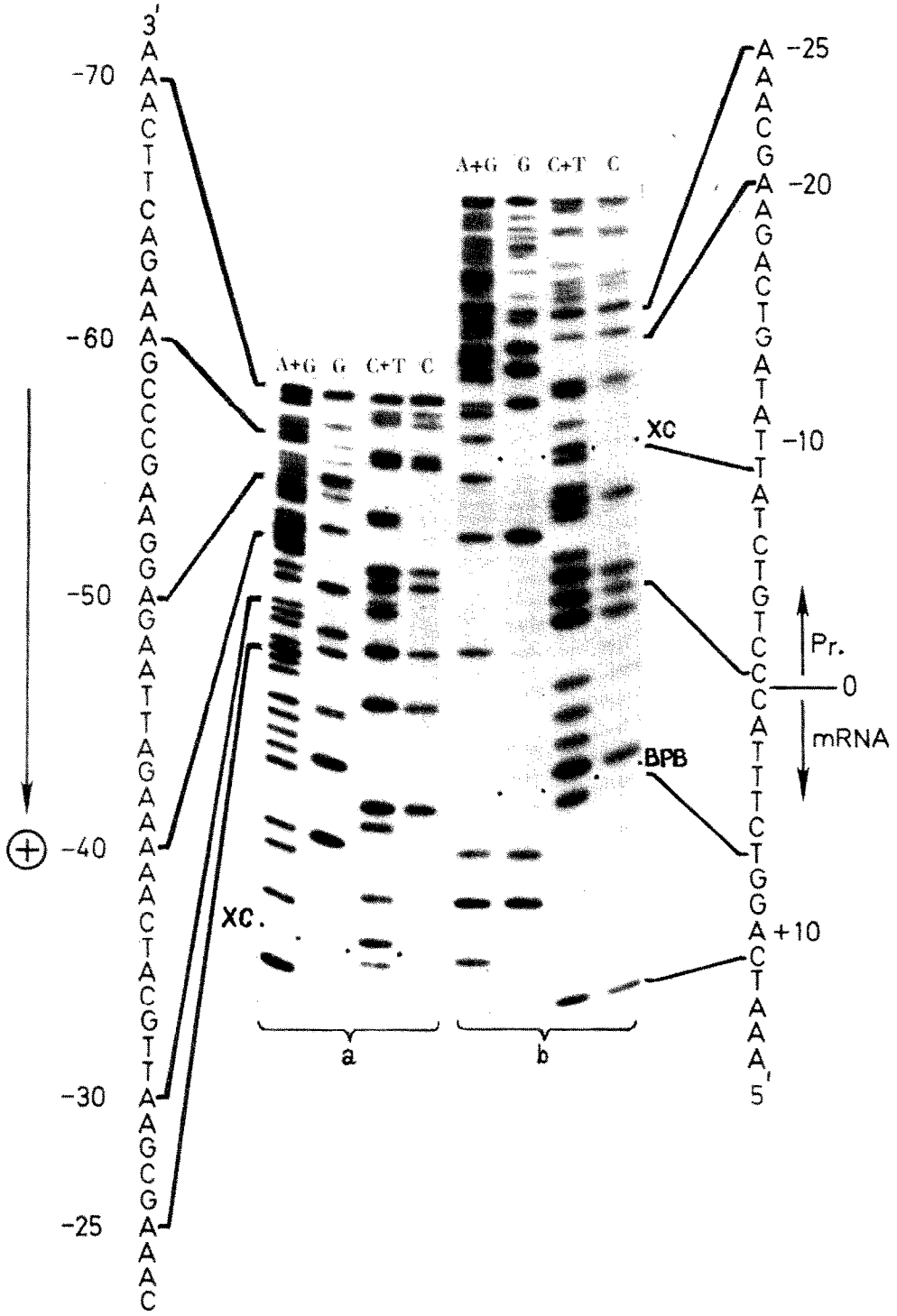
3'
A
A
A
C
T
T
C
A
G
A
A
A
G
C
C
C
C
G
A
A
G
G
A
G
A
A
T
T
A
G
A
A
A
A
A
C
T
A
C
G
T
T
A
A
G
C
G
A
A
A
C
-70
-60
-50
-40
-30
-25
A+G G C+T C
A+G G C+T C
XC.
a
b
A -25
A
A
C
G
A -20
A
G
A
C
T
G
A
T
A
T
T
A
T
C
T
G
T
C
C
C
A
T
T
T
C
T
G
G
A +10
C
T
A
A
A
5'
XC
-10
Pr.
0
mRNA
BPB

Fig.3

modification of the Maxam–Gilbert method [14–16] (fig.3).

The second method for synthesis of DNA segments with specific nucleotide sequences was by controlled T_4 DNA polymerase-mediated elongation of a synthetic oligonucleotide primer on a single-stranded template in the presence of an oligonucleotide-'stopper' for terminating the newly formed chain at the required length. The method takes advantage of the fact that T_4 DNA polymerase, contrary to *E. coli* DNA polymerase I, does not possess 5′→3′ exonuclease activity and is incapable of utilizing double-stranded DNA with a single-stranded nick as template-primer [23]. Figure 4 shows the ways of synthesizing the promoter region by this method which can be termed limited copying. Thus, to obtain the duplex (A–E) (way I), the 5′-^{32}P-labeled segment (A) and unlabeled segment (F) [with (A) in 4-fold excess] were complexed with single-stranded phage fd DNA, after which enzymatic synthesis of the complementary minus strand was carried out in the presence of phage T_4 DNA polymerase and four deoxyribonucleoside triphosphates. Both primers were elongated, the lengthening process of oligonucleotide (A) terminating after the 5′-end of segment (F), which played the part of a unique 'stopper', was reached. The resulting single-stranded 56-mer was isolated by electrophoresis in 8% polyacrylamide gel under conditions described for separating the strands of DNA fragments [14], annealed with a new portion of fd DNA [(+)-strand] and after treatment with endonuclease S_1 the (A–E) duplex obtained was isolated by gel filtration through Bio-Gel A-1.5 m.

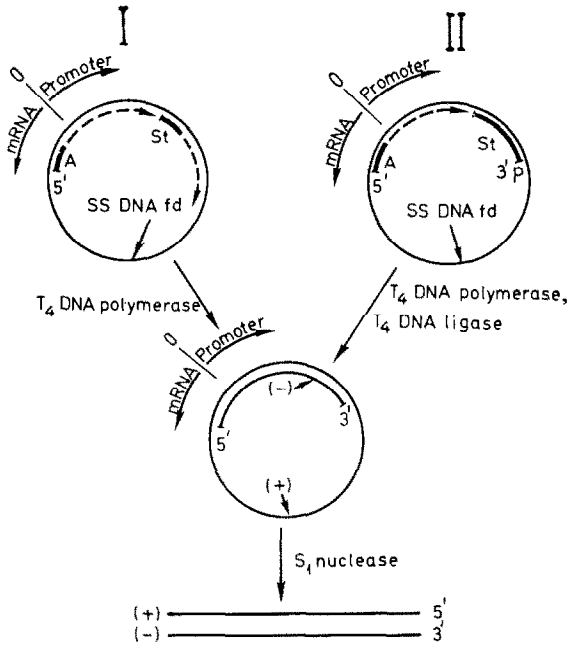

Fig.4. The plan for the synthesis of the promoter region by the limited template copying method. (A), segment-primer; (St), 'stopper' for way I (F), or for way II (F–H).

When 3′-blocked (e.g., phosphorylated) either oligo- or polynucleotide that is not a DNA polymerase substrate is used as a 'stopper' it can be incorporated into a synthesized duplex (way II, fig.4). We have shown the effectiveness of this method by its use in a second synthesis of the duplex (A–H). In this case the single-stranded 30-mer (F–H) with a 3′-phosphate group (inserted by means of terminal deoxynucleotidyl transferase [24]), formed by joining the segments (E), (G) and (H), was used as the 'stopper'. This 'stopper' and the 5′-^{32}P-labeled segment (A) were hybridized with a SS phage fd DNA and incubated with T_4 DNA polymerase and T_4 DNA ligase in the presence of the four deoxyribonucleoside triphosphates and ATP. Since the 'stopper' (F–H) was not elongated thereby, the double-stranded fragment (A–H) was obtained after treatment with S_1 nuclease. The lengths of the duplexes obtained were confirmed by comparing their mobilities in electrophoresis on polyacrylamide gel with those of segments obtained by means of ligase coupling, whereas the structure of the duplexes was confirmed by the Maxam–Gilbert method.

In developing methods for synthesizing double-stranded DNA with specific base sequences we attempted to shorten as much as possible the cumbersome chemical stages of the synthesis. From the

Fig.3. Autoradiograph of a sequencing gel of the minus strand of a 86-base-pair fragment (A–H). Electrophoresis was carried out on 20% polyacrylamide gel containing 7 M urea (slab 20 × 40 × 0.1 cm) at 1000 V and 20 mA: (a), for 16 h; (b), for 8 h. XC, xylene cyanol dye; BPB, bromophenol blue dye.

standpoint of its application in the study of the nucleic acid–protein interaction the methods described here on the one hand considerably facilitate determination of the size of the promoter region, and on the other hand provide wide possibilities for the modification of the promoter, in particular for the insertion into this segment of photosensitive nucleotide analogs, which are planned to be used further in the structure–functional study of *E. coli* RNA polymerase.

Acknowledgements

The authors are grateful to G. M. Dolganov and M. F. Shemyakin for their aid in isolating a number of enzymes, and to N. N. Modyanov, V. M. Lipkin and E. D. Sverdlov for helpful discussion of the results.

References

[1] Gilbert, W. (1976) in: RNA polymerase (Losick, R. and Chamberlin, M. eds) pp. 193–205, Cold Spring Harbor Labs, New York.
[2] Pribnow, D. (1975) Proc. Natl. Acad. Sci. USA 72, 784–788.
[3] Okamoto, T., Sugimoto, K., Sugisaki, H. and Takanami, M. (1977) Nucl. Acids Res. 4, 2213–2222.
[4] Brown, E. L., Belagaja, R., Fritz, H.-J., Fritz, R. H. and Norris, K. (1977) Fed. Proc. Fed. Am. Soc. Exp. Biol. 36, 732.
[5] Frischauf, A. M. and Scheit, K. H. (1973) Biochem. Biophys. Res. Commun. 53, 1227–1233.
[6] Sverdlov, E. D., Tsarev, S. A., Modyanov, N. N., Lipkin, V. M., Grachev, M. A., Zaychikov, E. F. and Pletnev, A. G. (1978) Bioorg. Khim. 4, 1278–1280.
[7] Ovchinnikov, Yu. A., Lipkin, V. M., Modyanov, N. N., Chertov, O. Yu. and Smirnov, Yu. V. (1977) FEBS Lett. 76, 108–111.
[8] Sugimoto, K., Sugisaki, H., Okamoto, T. and Takanami, M. (1975) Nucl. Acids Res. 2, 2091–2100.
[9] Ovchinnikov, Yu. A., Efimov, V. A. and Chakhmakhcheva, O. G. (1979) Bioorg. Khim. 5, 138–144.
[10] Schaller, H., Gray, C. and Herrmann, K. (1975) Proc. Natl. Acad. Sci. USA 72, 737–741.
[11] Panet, A., Van de Sande, J. H., Loewen, P. C., Khorana, H. G., Raae, A. J., Lillehaug, J. R. and Kleppe, K. (1973) Biochemistry 12, 5045–5050.
[12] Marvin, D. A. and Schaller, H. (1966) J. Mol. Biol. 15, 1–7.
[13] Seeburg, P. H. and Schaller, H. (1975) J. Mol. Biol. 92, 261–277.
[14] Gilbert, W. and Maxam, A. (1977) Proc. Natl. Acad. Sci. USA 74, 560–564.
[15] Skryabin, K. G., Zakharjev, V. M. and Baev, A. A. (1978) Dokl. Akad. Nauk SSSR 241, 488–490.
[16] Korobko, V. G. and Grachev, S. A. (1977) Bioorg. Khim. 3, 1420–1422.
[17] Van de Sande, J. H., Caruthers, M. H., Kumar, A. and Khorana, H. G. (1976) J. Biol. Chem. 251, 571–586.
[18] Richardson, C. (1965) Proc. Natl. Acad. Sci. USA 54, 158–165.
[19] Sanger, F. (1973) in: Virus Research (Fox, C. F. and Robinson, W. S. eds) pp. 573–599, Academic Press, London, New York.
[20] Sgaramella, V. and Khorana, H. G. (1972) J. Mol. Biol. 72, 427–444.
[21] Besmer, P., Miller, R. C., Caruthers, M. H., Kumar, A., Minamoto, K., Van de Sande, J. H., Sidorova, N. and Khorana, H. G. (1972) J. Mol. Biol. 72, 503–522.
[22] Sekiya, T., Takeya, T., Contreras, R., Küpper, H., Khorana, H. G. and Landy, A. (1976) in: RNA Polymerase (Losik, R. and Chamberlin, M. eds) pp. 455–472, Cold Spring Harbor Lab.
[23] Goulian, M., Lucas, Z. J. and Kornberg, A. (1968) J. Biol. Chem. 243, 627–638.
[24] Roychoudhury, R., Jay, E. and Wu, R. (1976) Nucl. Acids Res. 3, 863–878.