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Sequences of Two Restriction Fragments Containing Promoters of Bacteriophage fd.

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In order to define the structure of DNA specifying the initiation of transcription (promoter), two restriction fragments each containing a strong promoter were isolated from bacteriophage fd replicative form DNA by the use of restriction endonucleases. One named *HapD-Hga*1 was about 200 base pairs long and the other named *HapC-Hae*1 was about 300 base pairs long.

The fragments were phosphorylated at the 5′-termini with polynucleotide kinase and (γ^{32}P)ATP. Following partial digestion either by an enzymatic method or by a chemical method, the sequences from the 5′-ends were determined, and the total sequences of the two restriction fragments were deduced in conjunction with the sequence information previously obtained from their transcripts.

By comparing the two promoter sequences where the RNA initiation sites in the sequences are aligned, a unique sequence TATAAT was found to locate around the tenth nucleotide upstream from the RNA initiation site. This TATAAT sequence is also contained in the corresponding region of other high level promoters, lac UV5 and SV40 DNA. It was concluded that the TATAAT sequence has an important role for the function of high level promoter.

INTRODUCTION

A promoter is a start signal at the beginning of a gene or a gene cluster that directs the RNA polymerase to initiate RNA synthesis. Since genetic information is contained in a nucleotide sequence, a particular sequence must construct the promoter and the specific binding of RNA polymerase to this region allows the polymerase to initiate RNA synthesis. Analysis of the structure of the promoter is therefore essential for understanding of the molecular basis of gene expression. In earlier publications from this laboratory, RNA transcribed *in vitro* on bacteriophage fd relicative form (RF) DNA has been characterized.[1,2] The genome of this phage is a single-stranded circular DNA of about 1.7×10^6 daltons in molecular weight (about 6,000 bases). The quantity of this DNA is sufficient to code for eight phage-specific proteins.[3] Like other phages containing single-stranded DNA, a double-stranded replicative form DNA is formed upon infection of *E. coli* cells with this phage. The repicative form DNA occurs in two forms: one is the closed circle (RFI) and the other the open circle (RFII). When RNA is synthesized *in vitro* on RFI DNA, several different classes of RNA with unique starting sequences are synthesized from the minus strand of the template, that is complementary to the phage DNA.[2] The results indicate that RFI DNA provides sets of specific sites for initiation and termination of transcription. Since the transcription proceeds in the same direction and the longest RNA species has a size of about one turn of the template, a model for the fd transcrip-

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tion was proposed that RNA synthesis initiated at several different promoters proceeds in the same direction and terminates at a single unique site resulting different RNA classes.[4] In the meanwhile, restriction endonucleases which cleave DNA into unique fragments by recognizing specific sequences were isolated in this and other laboratories.[5,6] By the use of this type of enzyme, a physical map of this phage DNA was successfully constructed.[7] It was also found that cleavages by these enzymes do not destroy promoter function unless the cleavage site is located within promoter. Thus, the promoter regions were localized in the physical map of fd DNA by testing the template activity of each restriction fragment.[8]

The next attempt was focussed on the determination of the sequences in the promoter regions. Two high level promoters named G2 and G3 were chosen for the purpose, because the nature of these promoters was very similar: RNA polymerase binds tightly and initiates G-start RNA with almost identical efficiency. It is therefore expected that the sequence essential for promoter function is deduced as a common sequence. The detailed cleavage map in the region covering the G2 and G3 promoters is shown in Fig. 1. The G2 promoter is contained in a restriction fragment *HapD-Hga*I and the G3 promoter in a fragment *HapC-Hae*I (Fig. 1). The chain length of *HapD-Hga*I was estimated to be about 200 bases long and that of *HapC-Hae*I to be about 300 bases long, respectively, from their relative electrophoretic mobilities. When RNA was synthesized under the standard conditions, RNA of about 110 bases long (G2'RNA) was efficiently formed on *HapD-Hga*I by the termination of the G2 RNA species at the *Hga*I site, and RNA of about 210 bases long (G3'RNA) on *HapC-Hae*I by the termination of the G3 RNA species at the *Hae*III site, respectively (Fig. 1). Therefore, the sequence information needed for promoter function should be contained in the non-transcribed part (about 100 base pairs) of these fragments. In order to deduce the sequences of these fragments, the sequences of transcripts (G2'RNA and G3'RNA) were first determined, and then the sequences in the non-transcribed

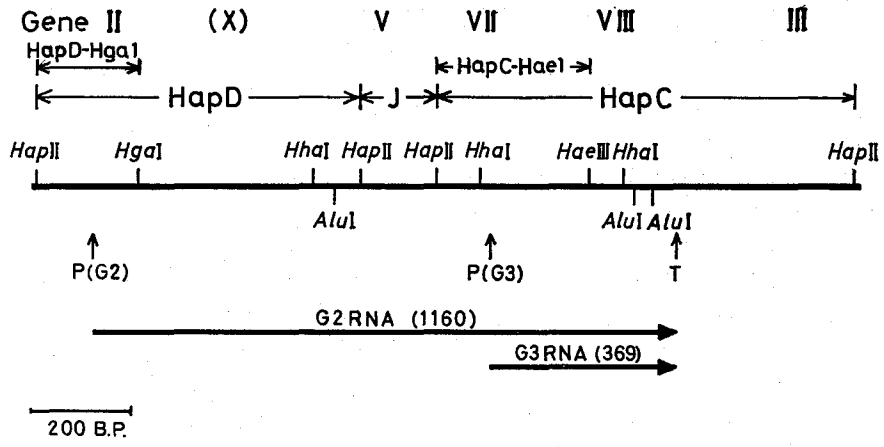

Fig. 1. Cleavage and transcription maps in the *HapD* to *HapC* region of bacteriophage fd.
Restriction endonucleases *Hap*II, *Hae*III, *Hga*I, and *Hha*I were prepared from *H. aphirophilus*, *H. aegyptius*, *H. gallinarum* and *H. haemolyticus*, respectively. Data were taken from ref. 8.

regions were analysed by transcribing them into RNA at specific reaction conditions.[9~11] In the course of this study, however, it was noted that the recognition sequences for restriction endonucleases used for preparation of the template were not identified in the sequences obtained for the terminal regions by the indirect method. This leads to an important question whether or not the sequence deduced by the RNA transcription method exactly reflects the sequence of the template.

In this paper, I re-examined the sequences of the two promoter containing fragments by direct DNA sequencing methods, and the exact sequences of these fragments were deduced in conjunction with the sequence information previously obtained from their transcripts.

MATERIALS AND METHODS

Restriction Fragments. fd RFI DNA uniformly labelled with ^{32}P was prepared from *Escherichia coli* K38 infected with phage fd, and digested with R·*Hap*II using the conditions previously described.[7] The digest was layered on 5% polyacrylamide gel columns (0.6 cm × 12 cm) formed in 34 mM tris–32mM KH_2PO_4–1 mM EDTA (pH 7.8) and electrophoresed for 16 hr at 2 mA/tube using the same buffer. The gels were covered with Saranwrap and autoradiographed. The *HapC* and *HapD* regions were cut out and homogenized and DNA was extracted with a few ml of 10 mM tris-HCl (pH 7.6) by incubation for overnight at 37°C. The extract was concentrated and desalted by passage through a Sephadex G50 column (0.4 cm × 15 cm). The fragment *HapC* was then digested with R·*Hae*III and the fragment *HapD* with R·*Hga*I, respectively. The digests were electrophoresed as described above, and the fragments *HapC-Hae*I and *HapD-Hga*I were extracted.

Enzymes. R·*Hap*II, R·*Hae*III, and R·*Hga*I were prepared from three *Haemophilus* strains, *H. aphirophilus*, *H. aegyptius* and *H. gallinarum*, respectively, as described in a previous paper.[5] Polynucleotide kinase was prepared from *E. coli* A19 infected with phage T4 amN82 according to the method Richardson,[12] and purified further by rechromatography on phospho-cellulose (Whatman P11) and DEAE-cellulose (Whatman DE52) columns. Alkaline phosphatase, venom phosphodiesterase and DNase I were purchased from Worthington Biochemicals.

Polynucleotide Kinase Labelling Procedure. About 10 pmoles of a restriction fragment were incubated with 10 μg of *E. coli* alkaline phosphatase (Worthington B.C., Grade BAPC) for 60 min at 55°C in 50μl of 10 mM tris-HCl (pH 8). 5 μl of 0.05 M EDTA (pH 8) were added and incubation was continued for an additional 20 min and heated for 3 min at 100°C to inactivate phosphatase activity. 400 pmoles of ($\gamma^{32}P$)ATP with a specific activity of about 1,000 Ci per mmole and 20 μl of 0.5 M tris-HCl (pH 8)–0.1 M $MgCl_2$–0.1 M 2-mercaptoethanol were added and the volume was adjusted to 200 μl with distilled water. 10 units of polynucleotide kinase in 10 μl were added and the mixture was incubated for 60 min at 37°C. The reaction was terminated by shaking with phenol. Phenol was removed by shaking with ethylether and the DNA fraction was isolated by passage through a Sephadex G100 column

(0.5 cm×5 cm).

Two-dimensional Fractionation Procedure Using Homochromatography. The(5′-^{32}P) restriction fragment was incubated at 37°C with 20 ng of DNase I (Worthington B.C.) and 0.2 μg of venom phosphodiesterase (Worthington B.C.) in 20 μl of 50 mM triethylamine bicarbonate (pH 8)–5 mM $MgCl_2$ containing 10 μg of sonicated calf thymus DNA as carrier. At 10 min intervals, 4 μl each was withdrawn, mixed with 50 μl of water, and immediately heated for 3 min at 100°C. The combined digests were dried *in vacuo* and dissolved in 2 μl of water. The sample was applied onto a cellulose-acetate strip (Schleicher & Schüll, 30 cm long) and electrophoresed for 50 min at 40 V/cm in a solution of 5% (v/v) acetic acid and 7 M urea, adjusted to pH 3.5 with a few drop of pyridine. The separated products were transferred onto a DEAE-cellulose thin layer plate (Macherey & Nagel, polygram CEL300–DEAE, 20 cm×20 cm) by the procedure of Southern.[13] The plate was washed with methanol for a few minutes and dried at room temperature. A filter paper (Whatman 3MM, 20 cm×20 cm) was clipped to the top of the plate and uniformly pressed by a plastic strip. The DEAE-cellulose plate was first developed with water up to 5 cm, and then for 10 hr at 60°C either with the homoxiture V (for analysis of mono- to deca-nucleotides) or with the homomixture III (for analysis of longer nucleotides). The homomixtures were prepared by hydrolysis of yeast RNA (Sigma, Tolura yeast RNA Type VI) with different concentrations of KOH as decribed by Jay *et al.*[14]

Sequencing by the Method of Maxam-Gilbert. The sequence determination by the method of A. Maxam and W. Gilbert[15] was performed following their protocol communicated to Dr.M. Takanami. The two labelled ends of the (5′-^{32}P) restriction fragment were separated either by secondary cleavage with a different enzyme or by strand separation. The fragments bearing a single terminal label were divided into four fractions. Two aliquots were reacted with dimethylsulfate and two aliquots with hydrazine under the conditions that an average one base at random position per strand reacts. The methylated bases were released from the polynucleotide backbone either by heating at neutral pH (7-methyl G) or by treatment at cold with 0.1 N HCl (3-methyl A). Hydrazinolysis of the pyrimidine were performed either in the presence of concentrated NaCl (C-specific) or in the absence of salt (pyrimidine specific). The destabilized phosphodiester bonds adjacent to the depurinated or depyrimidinated sites were then cleaved by heating in base (NaOH or piperidine). The four sets of partial chemical degradation products all beginning at the uniquely labelled 5′-end but terminating at various positions were analysed in parallel on 20% polyacrylamide slab gels using 50 mM tris-borate (pH 8.3)–1 mM EDTA.

Analysis of Nucleotides at the 5′-end. Oligonucleotides containing ^{32}P at the 5′-end were incubated for 30 min at 37°C with 20 ng of DNase I and 0.2 μg of venom phosphodiesterase in 20 μl of 50 mM triethylamine bicarbonate (pH 8)–5 mM $MgCl_2$. The digests were dried *in vacuo* and applied onto a polyethyleneimine (PEI)-cellulose thin layer plate (Macherey & Nagel) and developed with 0.5 M lithium formate (pH 3).

(γ^{32}P)ATP. (γ^{32}P)ATP was prepared by the method of Glynn and Chappell,[16] except that the exchange reaction was carried out in a reduced volume (25 μl) to increase its specific activity. The specific activity of (γ^{32} P)ATP prepared was about 1,000 Ci/mmole.

RESULTS

The Total Sequence of *HapC-Hae*1

Both the 5′-ends of *HapC-Hae*1 were highly labelled using (γ^{32}P)ATP and polynucleotide kinase. Since *HapC-Hae*1 contains an R·*Hha*I cleavage site (see Fig. 1), the two labelled ends were separated by secondary cleavage with R·*Hha*I, followed by gel electrophoresis. Each subfragment bearing a single terminal label was subjected to four sets of partial chemical degradation specific for each base, according to the method of Maxam and Gilbert.[15] The partial digests all containing the uniquely labelled 5′-end were then subjected to electrophoresis in parallel on polyacrylamide slab gels (45 cm long) capable of resolving products differing in length. The DNA sequence from the 5′-end was read directly from the resulting autoradiograms by evaluating the four tracks in parallel. The representative autoradiograms obtained for the left and right ends of *HapC-Hae*1 are shown in Fig. 2. The sequences from each 5′-end were read as indicated in the figure.

The sequence was also confirmed by analysis of partial enzymatic digests of the end labelled fragments. The fragments bearing a single terminal label were partially digested with a mixture of DNase I and venom phosphodiesterase, and fractionated by the two-dimensional fingerprinting procedure which uses homochromatography. A typical fingerprint obtained for the R·*Hap*II end is shown in Fig. 3. Since the direction and relative distance of migration of an oligonucleotide depends on the species of nucleotide added to the 3′-end, the sequence up to 20 to 25 nucleotides from the 5′-end was identified as indicated in figure.

The cleavage site specificity of R·*Hap*II and R·*Hae*III which were used for preparation of *HapC-Hae*1 has been determined (**Table** I).[6,18] Therefore, the sequences at both ends of *HapC-Hae*1 were deduced as in Fig. 4. The sequence of *HapC-Hae*1 has previously been analysed by the RNA transcription method.[11,17] Under the standard conditions for RNA synthesis, RNA synthesis was efficiently initiated at the G3 promoter. When RNA synthesis was carried out at a very low salt concentration, however, RNA synthesis was initiated at additional sites including terminal regions.[11] Thus, the sequence of the template was deduced from analysis of such low salt transcripts. The sequences at both ends of *HapC-Hae*1 obtained by the indirect method are shown in Fig. 4 for comparison. The sequence at the R·*Hap*II end obtained by both methods was identical. However, the sequence deduced for the R·*Hae*III end was different from the sequence obtained by the direct sequencing method. The sequence deduced by the indirect method contained seven extra-nucleotides at the right end, indicating that these extra nucleotides were incorporated into the 3′-end of the transcript under the conditions used for RNA synthesis. Although the mechanism involved in this reaction is quite unknown, it was noted that the sequence from position 199 to the 3′-end of the transcript is just complementary

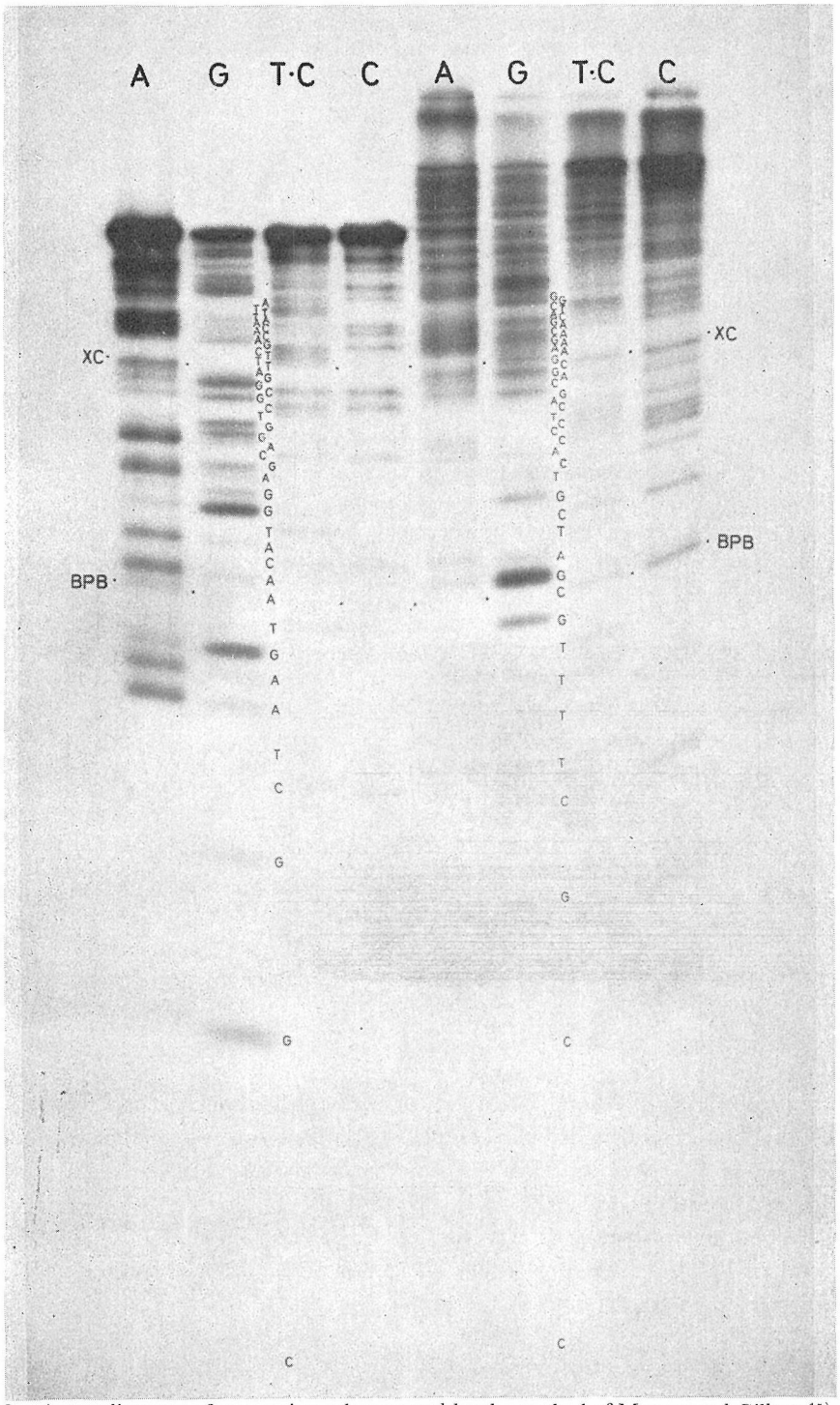

Fig. 2. Autoradiograms of sequencing gels prepared by the method of Maxam and Gilbert.[15]
Left four tracks are partial chemical digests derived from the *Hap*II end of *HapC-Hae*1 and right four tracks those derived from the *Hae*III end of *HapC-Hae*1. The sequences are read as indicated by the side of autoradiograms. According to the previous analysis,[17] the thick G-band apeared at the eleventh position from the *Hae* III end of *HapC-Hael* should consist of three G's.

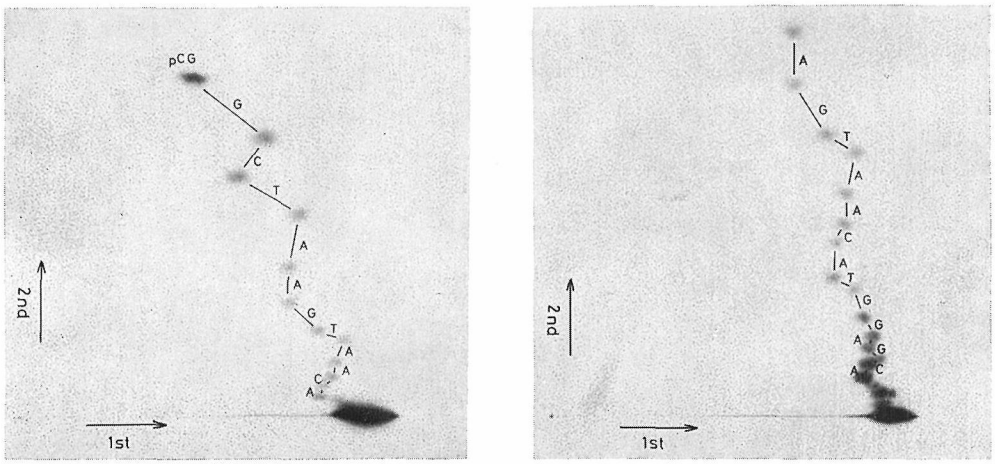

Fig. 3. Fingerprints of partial enzymatic digests derived from the *Hap*II end of *HapC-Hae*1.
Development in the first dimension was electrophoresis on a cellulose acetate film at pH 3.5 and that in the second dimension homochromatography on a DEAE-cellulose thin layer plate. The sequence is read as indicated in the fingerprint. The left was developed with homomixture V and the right with homomixture III.

Table I. Cleavage site specificity of restriction endonuclease R·*Hap*II, R·*Hae*III and R·*Hga*I
Data were taken from refs. 6, 18, 19.

R·*Hap*II	----pCpCpGpG ---- ---- GpGpCpCp----
R·*Hga*I	----pGpApCpGpCpNpNpNpNpNpNpNpNpNpN ---- ---- CpTpGpCpGpNpNpNpNpNpNpNpNpNpNp----
R·*Hae*III	----pGpGpCpC ---- ---- CpCpGpGp----

to the sequence from position 184 to 194 of the same strand. It is therefore likely that RNA polymerase switched the template strand at the end of the template and read back along the other strand of the template, as indicated in Fig. 4 (C).

The total sequence of *HapC-Hae*1 deduced in conjunction with the sequence information obtained by analysis of transcripts is shown in Fig. 5, in which nucleotides were numbered by reference to the first nucleotide of G3 RNA and those in the non-transcribed region were denoted by the minus sign.

The Total Sequence of *HapD-Hga*1

When *HapD-Hga*1 labelled at both the 5′-ends was partially digested by a mixture of DNase I and venom phosphodiesterase and analysed by the two-dimensional fingerprinting method, two series of oligonucleotides with increasing chain length were obtained as indicated in Fig. 6. Each spot was eluted and species of nucleotide

Fig. 4. The sequences deduced for the *Hap*II end (A) and the *Hae*III end (B) of *HapC-Hae*1.
The sequences previously obtained by the transcription method were indicated for comparison. Extra-nucleotides incorporated into the 3′-end of the transcript are boxed. The complementary sequences at the terminal region of the transcript are indicated by underlining. In (C), the possible base pairing at the terminal region of the transcript was indicated.

Fig. 5. The total sequence of *HapC-Hae*1.
Nucleotides were numbered by reference to the first nucleotide of G3 RNA and those in the non-transcribed region were denoted by the minus sign.

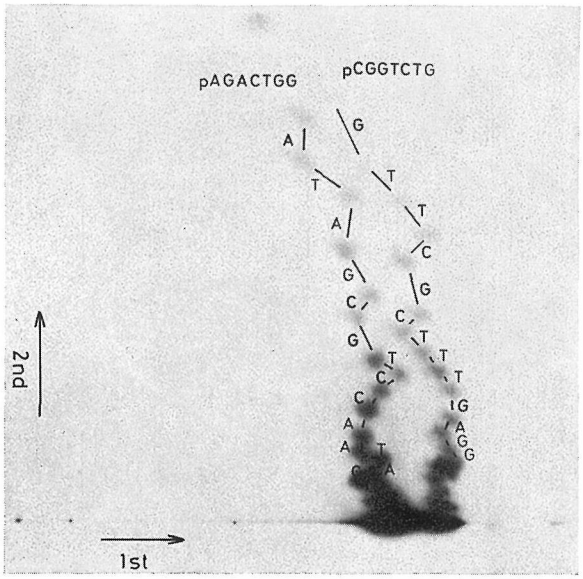

Fig. 6. A fingerprint of partial enzymatic digests derived from *HapD-Hga*I labelled at both 5′-ends.

Development in the first dimension was electrophoresis on a cellulose acetate film at pH 3.5 and that in the second dimension homochromatography on a DEAE-cellulose thin layer plate. One set of oligonucleotides contained C and the other A at the 5′-end. Those containing C at the 5′-ends were assigned to the *Hap*II end.

at the 5′-end was analysed. As the result, one set of oligonucleotides were found to contain C and the other A at the 5′-end. Since restriction fragments generated by R·*Hap*II should contain C at the 5′-end, the set of oligonucleotides containing C were assigned to those created at the R·*Hap*II site, and the other at the R·*Hga*I site. From the direction and relative distance of migration, the sequences up to 20 to 25 nucleotides from each 5′-end were identified as indicated in Fig. 6.

The sequences were also confirmed by the sequencing method of Maxam and Gilbert.[15] The end labelled *HapD-Hga*I was denatured in 0.3 N NaOH and strand separation was carried out by electrophoresis in polyacrylamide gels under denaturing conditions. Each strand was then subjected to partial chemical degradation and analysed as in the previous section. The sequence read from the resulting autoradiograms were identical to those obtained from the fingerprinting method (data not shown). Since the cleavage site specificity of R·*Hap*II and R·*Hga*I has been determined (Table I), the sequences at both ends of *HapD-Hga*I were deduced as in Fig. 7. In previous work from this laboratory[9,10], the sequence of *HapD-Hga*I has been analysed by the RNA transcription method. In Fig. 7 the sequences at the terminal regions obtained by the indirect method are shown for comparison. The sequence obtained for the R·*Hap*II end by both methods was identical. However, the sequence obtained for the R·*Hga*I end by the indirect method was again longer than that determined by the direct sequencing method, indicating that transcription of *HapD–Hga*I resulted in incorporation of additional nucleotides to the 3′-end. As indicated in Fig. 7 (C), the sequence at the 3′-end of the transcript is just complementary to

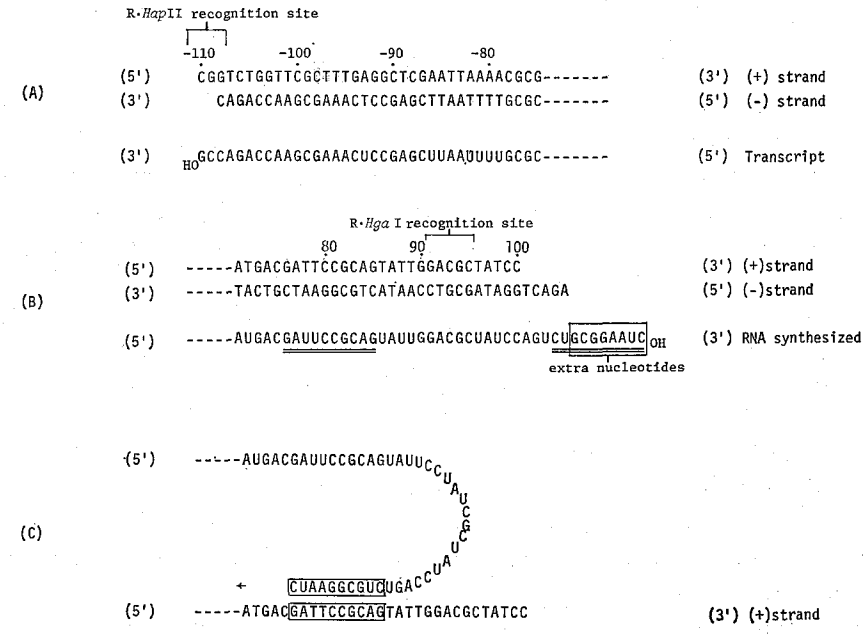

Fig. 7. The sequences deduced for the *Hap*II end (A) and the *Hga*I end (B) of *HapD-Hga*1.
The sequences previously obtained by the transcription method (9, 10) were indicated for comparison. Extra-nucleotides incorporated into the 3′-end of the transcript were boxed. The complementary sequences seen at the terminal region of the transcript were underlined. In (C), the possible base-pairing at the terminal region of the transcript was indicated.

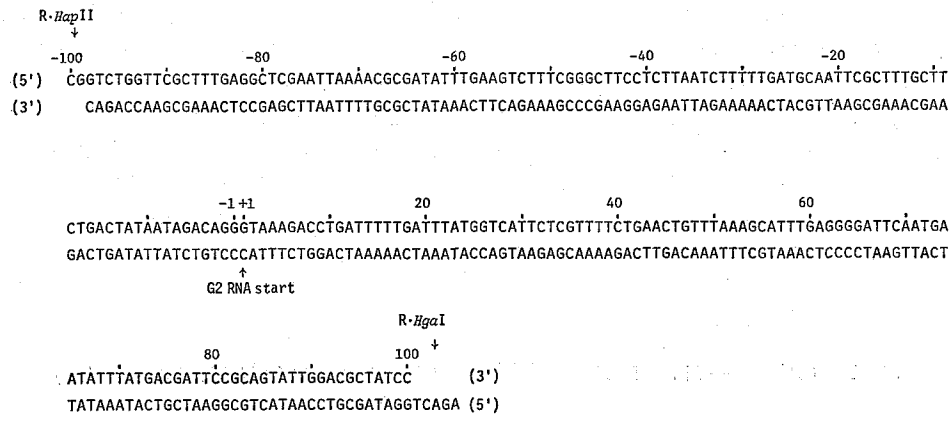

Fig. 8. The total sequence of *HapD-Hga*1.
Nucleotides were numbered by reference to the first nucleotide of G2 RNA and those in the non-transcribed region were denoted by the minus sign.

the sequence from position 75 to 85 of the same strand. It is therefore interpreted that RNA polymerase read back along the other strand at the end of the template, as observed with *HapC-Hae*1 (see Fig. 4C).

The complete sequence of *HapD-Hga*1 deduced in conjunction with the previous sequence information is shown in Fig. 8.

DISCUSSION

In previous studies from this laboratory,[9~11,17] the sequences of two-promoter containing fragments have been analysed by the RNA transcription method. However, the sequences obtained by this indirect method did not contain the recognition sequence for restriction endonucleases at the terminal region. In this paper, the sequences of these restriction fragments were analysed by the direct sequencing method, and it was found that the sequences previously deduced by the transcription method were longer than the fragments as template (see Fig. 4 and 7). This is obviously due to incorporation of extra-nucleotides to the 3'-end of the transcript. The mechanism involved in this region is quite unknown. As indicated in Fig. 4C and 7C, the sequence of extra-nucleotides is complementary to a region near the end of the same strand. It is therefore likely that RNA polymerase which reached the end of the template switched the template strand before running through the template and read back the other strand of the template. The question remains as to why the RNA chain did not grow longer, but this finding gives a warning to the sequence determination by the transcription method.

The complete sequences of two promoter-containing restriction fragments are shown in Fig. 5 and 8. All the sequence information needed for promoter function should be contained in the non-transcribed regions of the sequences. First the sequences of two promoter regions were compared with the initiation points vertically aligned and with transcription oriented from left to right. As the result, a unigue sequence TATAAT was found to be contained to the immediately left of the RNA initiation point in the non-transcribed region of the sequence (Fig. 9). The region where RNA polymerase forms a stable complex (RNA polymerase-binding site) has been characterized by the DNase digestion method of the complex.[9,20,21] The polymerase–binding site is about 45 base pairs long and contains the RNA initiation point at the center. Therefore, the TATAAT sequence is located in the non-transcribed part of the polymerase-binding site. It has previously been noted that the G2 promoter contains regions with two-fold rotational symmetry, with one located in the non-transcribed region of the polymerase-binding site.[9] The G3 promoter also contains

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fd G2    (5')  ---TTTGCTTCTGACTATAATAGACAGGGTAAAGACCTGATTTTTG---  (3')
                                        ↑
fd G3    (5')  ---TTTCGCGCTTGGTATAATCGCTGGGGTCAAAGATGAGTGTTTT---  (3')
                                      ↑
lac UV5  (5')  ---GCTTCCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAACAA---  (3')
                                      ↑
SV40     (5')  ---TTTATTGCAGCTTATAATGGTTACAAATAAAGCAATAGCATC----  (3')
                                      ↑
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Fig. 9. Comparison of the sequences of four high level promoters with vertically aligned with reference to the TATAAT sequence and with transcription oriented from left to right.

The TATAAT sequence was indicated in boxes and the RNA initiation site by arrows. The sequences of lac UV5 and SV40 DNA were taken from refs. 29 and 30, respectively.

some symmetrical regions but comparison of the symmetrical regions does not reveal a common pattern. So far, the sequences of ten different promoters including fd G2 and G3 have been determined.[22~30] By comparing these sequences, other promoters also contain the sequences generally homologous to TATAAT in the corresponding region, as originally noted by pribnow (ref. 20, Pribnow box). Gilbert's group has sequenced a few mutations of lac promoter that enhance the low level of promotion that occurs in the absence of the cap-protein.[29] As the result, they found that a promoter mutation UV5 which greatly increases promotion is a double change of GT to AA in the Pribnow box (ref. 29, TATGTT to TATAAT). The sequence in UV5 is therefore identical to the sequence found in two strong fd promoters. Another strong promoter found in SV40 DNA also contains this TATAAT sequence in the corresponding region.[30] Okamoto *et al.*[31] have recently shown by a substitution experiment with fd G3 promoter that no unique sequence is necessary in the region upstream from the TATAAT sequence. These lines of evidence strongly suggest that the sequence in the Pribnow box plays an indispensable role in promoter function and that TATAAT is a unique sequence for high level promoters.

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