ELIMINATION OF PROMOTER FUNCTION BY BASE MODIFICATION OF DNA

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1. Introduction

Promoters are DNA regions at which highly selective RNA polymerase binding and RNA chain initiation take place. They are not characterized by a unique primary structure. Comparison of published promoter sequences [1–14] reveals that there is not even an absolute requirement for a particular base at any position within such a region.

In view of this situation we started to examine the dependence of promoter function on specific structural elements of the nucleobases. Thus we 'removed' the 5-methyl of deoxythymidine (T_d) by replacement of this nucleoside by deoxyuridine (U_d). The modification was introduced into the codogenic strand of *Escherichia coli* phage fd RF DNA. Individual promoters were separated by cleavage of the circular DNA with restriction endonuclease *Hpa*II and polyacrylamide gel electrophoresis. *E. coli* RNA polymerase binding assays in the absence and presence of ribonucleoside triphosphates revealed that upon the DNA modification described the fd gene II promoter loses its affinity for the enzyme.

2. Materials and methods

DNA polymerase I of *E. coli* was isolated by H. Müller following the procedure in [15]. The large fragment of DNA polymerase I obtained by subtilisin treatment of the enzyme [16] was purchased from Boehringer Mannheim. *E. coli* DNA ligase was purified according to [17]. T4 DNA ligase was isolated by R. Frank and M. Wippermann as in [18]. *Hpa*II restriction endonuclease from *Haemophilus parainfluenzae* was purchased from Miles Labs, Elkhart, IN. *E. coli* RNA polymerase holoenzyme was a generous gift of Professor W. Zillig, Martinsried. fd-infected *E. coli* K12 Hfr 3300 was grown and fd DNA was isolated essentially as in [19,20] with the modification that phages were purified by two successive precipitations with 3% PEG in 0.5 M NaCl [21]. fd-specific oligonucleotide primers of chain length 11–13 originating from a DNase digest of fd RF DNA were prepared and purified by D. Müller. Ribo- and deoxyribonucleoside triphosphates were from Boehringer Mannheim. [α-^{32}P]A_dTP was purchased from the Radiochemical Centre, Amersham. RF DNA was synthesized in vitro in the presence of [α-^{32}P]A_dTP. Synthesis and isolation were done essentially as in [22]. 100% substitution of T_d by U_d within the codogenic strand was achieved by replacement of T_dTP by U_dTP and of DNA polymerase I by its large fragment. Detailed descriptions of these procedures will be published elsewhere (in preparation). For fragmentation of fd RF, 10 μg normal RF or 5 μg modified RF were incubated with 15 units or 25 units, respectively, of *Hpa*II at 37°C for 10 h in 0.5 ml of 30 mM Tris · HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTE, 3.5% glycerol. The fragments were deproteinized by 2 phenol extractions and desalted on Sephadex G-50. RNA polymerase binding experiments were performed in 20 mM Tris · HCl (pH 8.0), 10 mM $MgCl_2$, 120 mM KCl, 0.1 mM DTE, 0.1 mM EDTA, 5% glycerol at 37°C [23]. To allow RNA chain initiation GTP, ATP and UTP were present in some experiments at 0.1 mM

Abbreviations: RF DNA, replicative form DNA of bacteriophage fd; PEG, polyethyleneglycol; DTE, dithioerythritol; deoxyribonucleosides are marked by $_d$ following the nucleoside symbol

each RF was 0 15 pmol/ml RNA polymerase RF ratio was 20 Aliquots withdrawn from the reaction mixture were added to solutions containing denatured calf thymus DNA, 5 min later they were filtered through cellulosenitrate (MF 15 from Sartorius, Gottingen) and the filter bound material was eluted [23,24] When RNA chain initiation was measured filters were washed with ice-cold binding buffer (see above) containing 1 M KCl [25] followed by binding buffer without KCl DNA fragments eluted from the filters were separated on 1 mm thick 3 5% polyacrylamide/7 M urea slab gels equilibrated with 50 mM Tris H_3BO_3, 1 mM EDTA (pH 8 3) [26] and run at 20 V/cm The frozen gels were analyzed by autoradiography using Kodak X-Omat R films

3 Results and discussion

Starting from viral single-stranded fd DNA a modified RF DNA in which all T_d-moieties of the codogenic strand were replaced by U_d was synthesized in an oligonucleotide-primed 'repair' synthesis [22,27] catalyzed by the large fragment of *E coli* DNA polymerase I lacking the 5′-exonuclease function [16] Open circular RF II species were converted to covalently closed RF I by *E coli* or T4 DNA ligase

The modified DNA was cleaved by restriction endonuclease *Hpa*II from *Haemophilus parainfluenza*. This allows discrimination between the interactions of different fd promoters with *E coli* RNA polymerase [23]

The purified *Hpa*II fragments were incubated with RNA polymerase holoenzyme at 37°C and 120 mM KCl These conditions were shown to allow only specific binding of the enzyme to promoter regions [23] After 20 s, 50 s, 2 min and 5 min, aliquots were withdrawn and examined by a filter binding assay which traps RNA polymerase and DNA complexed by the enzyme [23,24] *Hpa*II fragments eluted from the filters were separated by polyacrylamide gel electrophoresis Assays with unmodified DNA were run as control

Figure 1 shows the fragments of normal and U_d-substituted fd RF DNA bound by RNA polymerase after a 5 min incubation at an enzyme RF ratio of ~20 Both fragment patterns are qualitatively identical with the exception of fragment H carrying the fd gene II promoter as the only RNA polymerase binding site

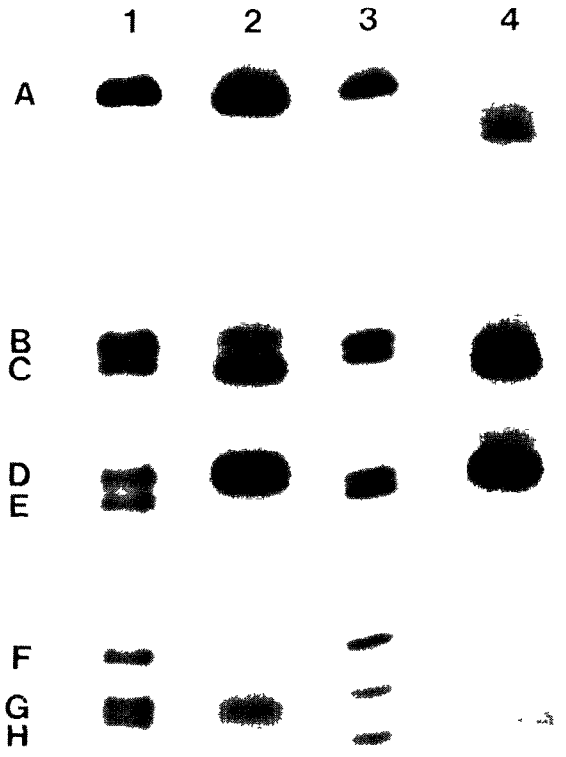

Fig 1 *Hpa*II fragments of normal and deoxyuridine-containing fd RF DNA bound by RNA polymerase ^{32}P-labeled fd RF DNAs (37 5 pmol) pre-fragmented by *Hpa*II were incubated with 750 pmol RNA polymerase holoenzyme in 250 μl for 5 min at 37°C The reaction was stopped by addition of excess denatured calf thymus DNA Fragments bound by RNA polymerase were trapped on cellulosenitrate filters, separated on polyacrylamide gels and visualized by autoradiography For details see section 2 Results of such analyses are shown in slots 2 (normal DNA) and 4 (U_d-containing DNA) Slots 1 and 3 show complete sets of *Hpa*II fragments A–H containing T_d or U_d, respectively

Obviously in the case of U_d-substitution this fragment does not form a complex with the enzyme This result also holds for the samples withdrawn earlier from the reaction mixture As can be expected promoter binding is a rather fast reaction Half-maximal binding of the fastest fd promoters (of genes X, II and VIII) requires 15–60 s under the conditions applied, and there is no promoter-containing fragment that is not at least partially bound after only 20 s

(unpublished results). On the other hand the half-life of the gene II promoter–RNA polymerase complex was found to be in the range of 30 s [28]. Though rather unlikely there might exist the possibility that the modified promoter forms a complex too short-lived to be detected by our assay, but long-lived enough to allow RNA chain initiation, which is known to occur in <1 s after RNA polymerase binding [28]. So the binding experiment was repeated in the presence of 3 ribonucleoside triphosphates. Under these conditions a stable ternary complex of unmodified fragment H, RNA polymerase and a short RNA chain is formed (unpublished results). We were unable, however, to find such a complex with U_d-substituted fragment H. From these results we conclude that the T_d analogue eliminates the function of the gene II promoter, most probably by preventing RNA polymerase binding.

As mentioned before such a drastic effect was not observed with the other U_d-modified fd promoters. There are several aspects in which the normally strong gene II promoter differs from the other strong phage promoters. From comparison of the sequences (fig.2) it is obvious that most of the U_d-moieties are found not only at different positions but also next to different neighbouring nucleotides. Moreover the gene II promoter region is unusually rich in A_dT_d/U_d (~78%) and has a high U_d content as roughly 42% of the base pairs are A_dU_d pairs.

Based on crystallographic data for U_dMP (C1′–exo puckering of deoxyribose and tg conformation about the C4′–C5′ bond), which differ considerably from those obtained for T_dMP as well as for UMP, it has been argued that replacement of T_d by U_d would distort the normal stacking, hydrogen bonding, and backbone geometry of the double helix [31]. In fact a T_m decrease of poly A_dU_d [32] and U_d-containing DNA [33] relative to their normal counterparts has been observed and proves a less stable structure. Moreover the altered electrophoretic mobility of U_d-substituted fragments in polyacrylamide gels (see fig.1) indicates conformational changes within several regions of the fd genome at least under certain conditions. So it seems that the 5-methyl of T_d plays a rather important role for DNA structure, and the elimination of promoter function reported could reflect a structural alteration of the highly U_d-substituted gene II promoter region. Rather long A_dT_d/U_d-rich self-complementary sequences exist in this part of the phage genome, which could give rise to stem–loop-like structures. The stability of such a structure relative, e.g., to a double helical structure or to another stem–loop structure could be crucially altered by conformational strains brought about by U_d incorporation.

Apart from this interpretation the loss of promoter function could also be due to the lack of methyl groups in one or a few crucial positions being important contact sites for RNA polymerase. Following this hypothesis it seems rather unlikely that (all) such

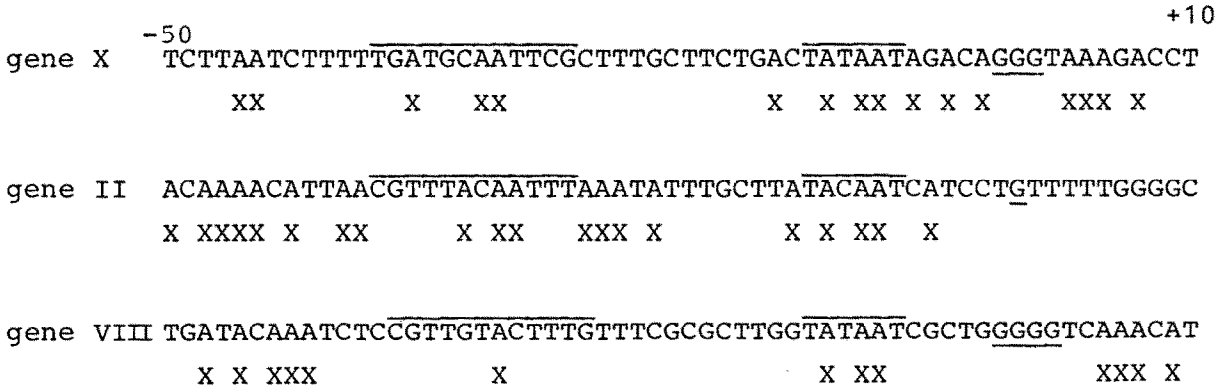

Fig.2. Comparison of the fd gene II promoter sequence to the promoter regions of fd genes X and VIII [2]. Viral strands are shown in 5′–3′ polarity. Similar sequences [29,30] are overlined. Transcriptional start sites are underlined. Sites of complementary strand modification are marked by X.

positions are situated within the Pribnow sequence [29] as promoters exhibiting identical modifications of this region like the gene X and gene VII promoters (see fig 2) show no or only a small decrease of promoter strength (our unpublished results)

For a precise localization of the effect described here studies with partially modified gene II promoter regions which can be obtained by use of unique primer molecules for DNA polymerase I should be quite useful

References

[1] Sekiya T, Gait, M J, Norris, K, Ramamoorthy, B and Khorana, H G (1976) J Biol Chem 251, 4481–4489

[2] Schaller, H, Beck, E and Takanami, M (1978) in Single Stranded DNA Phages (Denhardt, D. T et al eds) Cold Spring Harbor Laboratory, New York

[3] Dhar, R, Weissman, S M, Zain, B S, Pan, J and Lewis, A M, jr (1974) Nucl Acids Res 1, 595–613

[4] Pribnow, D (1975) J Mol Biol 99, 419–443

[5] Dickson, R C, Abelson, J, Barnes, W M and Reznikow, W S (1975) Science 187, 27–35

[6] Ptashne, M, Backmann, K, Hamayun, M Z, Jeffrey, A, Maurer, R, Meyer, B and Sauer, R T (1976) Science 194, 156–161

[7] Walz, A, Pirrotta, V and Ineichen, K (1976) Nature 262, 665–669

[8] Scherer, G, Hobom, G and Kossel, H (1977) Nature 265, 117–121

[9] Musso, R, DiLauro, R, Rosenberg, M and De Crombrugghe, B (1977) Proc Natl Acad Sci USA 74, 106–110

[10] Bennett, G N, Schweingruber, M E, Brown, K D, Squires, C and Yanofsky, C (1978) J Mol Biol 121, 113–137

[11] Bennett, G N, Brown, K D and Yanofsky, C (1978) J Mol Biol 121, 139–152

[12] Sanger, F, Air, G M, Barrell, B G, Brown, N L, Coulson, A R, Fiddes, J C, Hutchison, C A, iii, Slocombe, P M and Smith, M (1977) Nature 265, 687–695

[13] Calos, M P (1978) Nature 274, 762–765

[14] Weiher, H, v Gabain, A, Schaller, H and Bujard, H (1979) Z Physiol Chem 360, 397

[15] Englund, P T (1971) Proc Nucl Acid Res 2, 864–874

[16] Jacobsen, H, Klenow, H and Overgaard-Hansen, K (1974) Eur J Biochem 45, 623–627

[17] Olivera, B M and Lehmann, I R (1967) Proc Natl Acad Sci USA 57, 1426–1433

[18] Blocker, H, Frank, R and Koster, H (1978) Liebigs Ann Chem 991–1006

[19] Marvin, D A and Schaller, H (1966) J Mol Biol 15, 1–7

[20] Schaller, H (1969) J Mol Biol 44, 435–444

[21] Yamamoto, K R, Alberts, B M, Benzinger, R, Lawhorne, L and Treiber, G (1970) Virology 40, 734–744

[22] Gray, C P, Sommer, R, Polke, C, Beck, E and Schaller, H (1978) Proc Natl Acad Sci USA 75, 50–53

[23] Seeburg, P H and Schaller, H (1975) J Mol Biol 92, 261–277

[24] Jones, O W and Berg, P (1966) J Mol Biol 22, 199–209

[25] Richardson, J P (1966) J Mol Biol 21, 115–127

[26] Maxam, A M and Gilbert, W (1977) Proc Natl Acad Sci USA 74, 560–564

[27] Goulian, M and Kornberg, A (1967) Proc Natl Acad Sci USA 58, 1723–1730

[28] Seeburg, P H, Nusslein, C and Schaller, H (1977) Eur J Biochem 74, 107–113

[29] Pribnow, D (1975) Proc Natl Acad Sci USA 72, 784–788

[30] Gilbert, W (1976) in RNA Polymerase (Losick, R and Chamberlin, M eds) pp 193–205, Cold Spring Harbor Laboratory, New York

[31] Viswamitra, M A, Seshadri, T P and Post, M L (1975) Nature 258, 542–544

[32] Chamberlin, M, Baldwin, R L and Berg, P (1963) J Mol Biol 7, 334–349

[33] Takahashi, I and Marmur, J (1963) Nature 197, 794–795