CONDITIONS AFFECTING DNA CLEAVAGE BY *Tth*I AT A *Tth*I ENDONUCLEASE–*dam* METHYLASE OVERLAPPING SEQUENCE

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1. Introduction

The *Escherichia coli dam* gene codes for a site-specific DNA methylase which methylates adenine in the sequence GATC at the N6 position of the purine ring [1]. The effect of this modification on the action of several restriction enzymes whose recognition sites are equal or include the methylated sequence depends on the endonuclease itself. For example, the enzymes *Mbo*I, *Bcl*I and *Dpn*II do not cleave a sequence containing $GA^{me6}TC$ [2–4], while *Dpn*I requires the presence of 6MeAde in GATC sites in order to cleave the DNA [4]. On the other hand, isoschizomers *Sau*3AI, *Fnu*EI and *Pfa*I actively hydrolyze sequences that have undergone adenine methylation [5–7]; *Sau*3AI, however, is inhibited by the methylation of cytosine of the indicated sequence [8].

Backman has recently reported that methylation of adenine residues can also render DNA resistant to cleavage when there is an overlap between the recognition sequence of a restriction enzyme and that of the *dam* methylase [9]. One of such cases is represented by the sequence $\frac{TCGATC}{AGCTAG}$ present in pBR322 at 1126 base pairs from the *Eco*RI site [10], in which the *Taq*I (TCGA) [11] and *dam* (GATC) recognition sequences overlap.

Since this latter phenomenon may lead to erroneouis observations when mapping genomes or during sequencing experiments, we have decided to characterize it further. Working with the enzyme *Tth*I, an isoschizomer of *Taq*I isolated from *Thermus thermophilus* HB8 [12,13], we have found conditions that either increase or decrease susceptibility to DNA cleavage at the *Tth*I–*dam* methylase overlapping sequence.

Abbreviations: A^{me6} or 6-MeAde, C^{6}-methyladenine; C^{me5}, methylcytosine

2. Materials and methods

2.1. *Enzyme purification*

*Tth*I endonuclease was purified from *Thermus thermophilus* HB8 following the procedure described by Venegas et al. [13]. At 2 μg, this enzyme preparation produced complete digestion of 1 μg λ-DNA in 60 min at 65°C.

2.2. *Assay of* Tth*I activity*

Digestions were carried out in either 20 mM Tris–HCl (pH 8.0) or 20 mM glycine buffer (pH 10.0), 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 50 mM NaCl, 1 μg of pBR322 DNA and 2 μg of *Tth*I preparation in a final volume of 20 μl. The samples were incubated for 60 min at 65°C and the reactions were halted with 10 μl solution containing 20% glycerol, 1% SDS, 50 mM EDTA and 0.05% bromophenol blue. The products were then analyzed by polyacrylamide gel electrophoresis as previously described [13].

2.3. *Preparations of DNAs*

Plasmid pBR322 DNA was prepared from *Escherichia coli* HB101 (*dam*$^+$) and *E. coli* GM48 (*dam*$^-$) strains using standard procedures.

3. Results

In agreement with Backman's observations [9], we found that digestion with *Tth*I restriction endonuclease of unmethylated pBR322 DNA obtained from *dam*$^-$ cells yields products resulting from cleavage at all recognition sites on pBR322 DNA (fig.1a). However, digestion of DNA methylated by growth in *dam*$^+$ cells reveals a site which is refractory to cleavage by

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the enzyme under the same conditions (fig.1b). This is the site in the pBR322 genome in which the *Tth*I and *dam* methylase recognition sequences overlap [10].

In order to render this particular site sensitive to endonucleolytic digestion, we tested the effect of increasing the enzyme concentration in the incubation mixture. As shown in fig.1, lanes c through e, a ten-fold excess of *Tth*I produces almost complete digestion of pBR322 DNA methylated by *dam*, although a certain amount of the fragment containing the overlapping sequence remains unhydrolyzed.

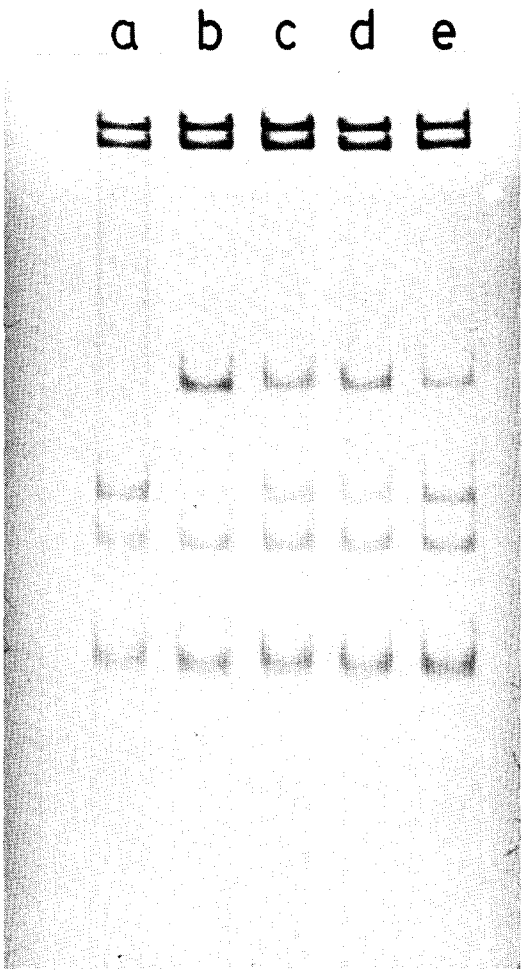

Fig.1. *Tth*I digestion of pBR322 DNA with different concentrations of enzyme. Lane a: unmethylated DNA obtained from *E. coli* GM48 (*dam*$^-$) incubated with 2 μg of enzyme preparation. Lanes b–e: methylated DNA obtained from *E. coli* HB101 (*dam*$^+$) incubated with 2,4,10 and 20 μg of enzyme preparation.

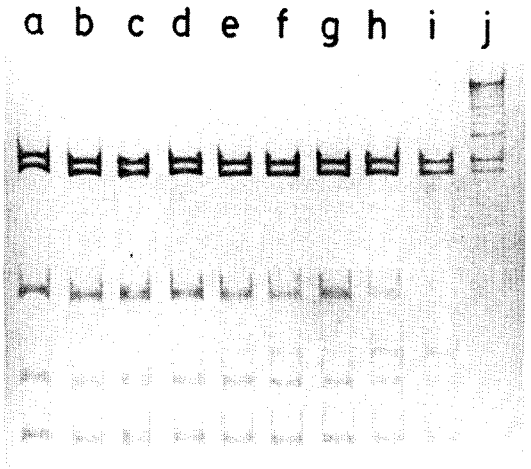

Fig.2. Effect of pH on digestion by *Tth*I of pBR322 DNA methylated by *dam*. Lanes a and b: potassium phosphate buffer pH 6.0 and 7.0. Lanes c–f: Tris–HCl buffer pH 7.5, 8.0, 8.5 and 9.0. Lanes g–j: Glycine buffer pH 9.0, 9.5, 10.0 and 10.5.

Analysis of the pH optimum for *Tth*I activity utilizing pBR322 as a substrate indicated that this enzyme is active over a wide range of pH, and that complete digestion by *Tth*I at the refractory site could be attained around pH 10.0. Moreover, as can

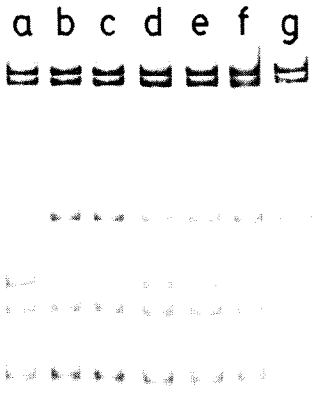

Fig.3. Digestion of pBR322 DNA by *Tth*I under different experimental conditions. All incubations were carried out at pH 10.0. Lane a: standard conditions; lane b: 30 mM $MgCl_2$; lane c: 2 mM $MnCl_2$; lane d: incubation during 30 min; lane e: incubation at 55°C; lane f and g: assays contain 3 M urea and 10% formamide, respectively.

be seen in fig.2, cleavage at this site increases gradually when the pH of the assay is raised from 7.5 to 10.0. Nevertheless, to obtain complete digestion at pH 10.0, the assay conditions described in section 2 must remain unaltered. Thus, if the salt concentration (fig.3, lanes b and c) and either the time (fig.3d) or the temperature of incubation (fig.3e) are varied, the overlapping sequence is not cleaved by *Tth*I. The same effect is obtained when denaturing agents like urea or formamide are added to the assay mixture (fig.3f and g). As it can be seen in fig.3, in spite of these changes in the incubation conditions, the rest of the TCGA sites present in the *dam*-methylated pBR322 DNA substrate are cleaved quantitatively.

4. Discussion

Chemical modification of DNA at a sequence recognized by a restriction enzyme may be inhibitory to digestion by the endonuclease, depending on the base which is methylated. Thus *Tth*I, which cleaves the DNA at the sequence TCGA, is inhibited by methylation in both strands at the N6 position of adenine [14], while the $TC^{me5}GA$ sequence retains its sensitivity to double-strand digestion by *Taq*I [8].

Methylation of adenine in only one strand at TCGA sites also interferes with *Tth*I digestion [9]. In vivo this type of modification arises when the *dam*-methylase and *Tth*I recognition sequences overlap. The work described above demonstrates that this structure can be cleaved using alkaline pH. However, *Thermus thermophilus* DNA, which is methylated in both strands, remains unaffected when incubated at pH 10.0 with high concentrations of this enzyme (data not shown).

In digesting *dam*-modified pBR322 DNA, *Tth*I behaved in the same fashion as its isoschizomers *Taq*I and *Tfl*I [14,15]. With respect to other substrates, we found that a yeast *met* tRNA gene which we have cloned and sequenced (unpublished) and ColE1 DNA contain a *Tth*I recognition site in their sequence which is cleaved at pH 10.0 but not at pH 8.0 (data not shown). However, complete digestion of both DNAs at pH 8.0 is obtained when they are prepared from a *dam*$^-$ *E. coli* strain.

Plasmid pBR322 DNA also contains a $\frac{GAAGATC}{CTTCTAG}$ sequence in which *dam*-methylase and *Mbo*II (recognition sequence GAAGA) sites overlap [9,10]. We have found that high levels of *Mbo*II at pH 10.0 are unable to cleave the *dam*-modified sequence. The different behaviour that both restriction enzymes exhibit may be due to the fact that *Tth*I cleaves the DNA within the recognition sequence (T↓CGA), while *Mbo*II does it seven and eight nucleotides away $\left(\begin{smallmatrix}GAAGAN_8\downarrow\\CTTCTN_7\end{smallmatrix}\right)$ [7].

This work also demonstrates the useful properties of the restriction nucleases obtained from thermophilic bacteria, since these enzymes can function at temperatures around 70°C and also in the presence of denaturing agents.

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