Dam methyltransferase sites located within the loop region of the oligopurine-oligopyrimidine sequences capable of forming H-DNA are undermethylated *in vivo*

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ABSTRACT

Several derivatives of pUC18 plasmid were constructed that contained oligopurine-oligopyrimidine (pur-pyr) motifs surrounded by Dam methylation sites. Inserts of two of the molecules (pPP1 and pPP2) were able to adopt the triple-stranded conformation *in vitro* and show *in vivo* a remarkable undermethylation of specific sites when grown in JM105 *dam*$^+$ strain. Mapping experiments revealed that undermethylated GATC sequences were located exclusively within the single-stranded loop region of the sequence involved in H-DNA formation. Control molecules which either contained the pur-pyr tracts (pPPK and pKK42) or not (pUC18) and were not able to form the triple-stranded conformation were found to be normally methylated by the *dam* gene product *in vivo*. Location of GATC within the triplex forming sequence seems to be a prerequisite for achieving its *in vivo* undermethylation. *E.coli* host factors are involved in the observed phenomenon. This has been deduced from the fact that the undermethylated state of pPP1 and pPP2 does not depend on the phase of growth of host cells and is steadily maintained up to 50 hours, whereas the kinetics of Dam methylation *in vitro* of sites located within the triplex loop does not differ substantially from the kinetics of methylation of other sites on the vector. Full methylation can be readily achieved *in vitro*. Additional factor(s) that operate *in vivo* to control the undermethylated state are most likely proteins since the observed effect can be suppressed by chloramphenicol administration to the cell cultures.

INTRODUCTION

Certain pur-pyr sequences exposed to negative supercoiling or low pH are able to adopt an unusual structure named H-DNA (1–20). Considerable interest in the structural behavior of pur-pyr sequences is certainly related to their frequent occurrence in eucaryotic genomes near active genes (21–26) or close to the regions involved in recombination events (1,27–32). The most characteristic feature of H-form DNA is the triplex core which arise by disrupting approximately one half of the pur-pyr stretch and folding back the homopyrimidine strand down the major groove of the second half of the repeat. Thus the third (homopyrimidine) strand of the triplex core interacts with purines of the helical duplex by Hoogsteen base pair leading to T·A·T and C·G·C triads (for details of the model see Refs. 8,14,16,20 and 32). Structural nature of the second half of the purine strand which is not involved in the triplex core formation remains uncertain. Based on the chemical-modification data, recently we suggested that it might not be single-stranded but by interacting with the triplex core could instead form a tetraplex structure (19).

Involvement of H-DNA in the regulation of a number of biological processes has been implied (12,16–18,32). Direct evidence, however, for existence of H-DNA in the living cell is not yet available. The *in vivo* existence of some other examples of unusual conformations such as Z-DNA or cruciforms has been proven using suitable genetic probes (33–35). A similar approach was used in the studies presented in this paper in which we describe an unusually specific and low *in vivo* Dam methylation of GATC sequences placed within the loop region of the pur-pyr segment capable of forming H-DNA.

MATERIALS AND METHODS

Plasmids

All plasmids used in these studies are derivatives of pUC18 (36). Construction of pPP1 was described previously (19). pPP2 was made by cloning of two complementary synthetic oligonucleotides: $GATC(AG)_7ATCGATCG(AG)_7$ and $GATC(CT)_7CGATCGAT(CT)_7$ into *Bam*HI site of pUC18. pPPK plasmid derived from pPP2 by deletion of the *Cla*I-*Nar*I segment. The *Cla*I site is present on the synthetic insert of pPP2 within the spacer separating two $(AG)_7$ blocks, whereas *Nar*I site is located on the pUC18 vector. pKK42 was constructed by cloning of the 34 bp *Hpa*II fragment from pBR322 into *Cla*I site of pPP2. Plasmids were isolated either from JM105, *dam*$^+$ strain (36) or from GM2163, *dam*$^-$ strain (37). Topoisomeric samples were prepared as described before (38).

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Fig. 1. Plasmids used in these studies. The hatched boxes represent the pUC18 vector sequences flanking the inserts. Dam methylation sites adjacent to pur-pyr sequences are indicated by straight lines.

Chemical modifications

Chemical modifications with diethyl pyrocarbonate (DEPC) and OsO_4 were as described previously (12,19).

Assay for Dam methylation

The degree of methylation of GATC sites on plasmids *in vivo* was analyzed by isolation of DNA using Birnboim's method (39) and digestion with 100-fold excess of *Mbo*I (Pharmacia). The products were cut with *Ava*II and separated on agarose gels.

Methylation *in vitro* was performed as follows: 1 μg of DNA obtained from the *dam*$^-$ strain was placed in 50 mM Tris-HCl buffer, pH 7.5, 10 mM EDTA, 5 mM 2-mercaptoethanol, 80 μM S-adenosylmethionine. Reaction was initiated at 37°C by adding 10 units of Dam methylase (New England Biolabs). After an appropriate incubation time DNA was extracted with phenol followed by ether extraction (3 times), precipitation and digestion with excess of *Mbo*I and *Ava*II. Products were electrophoresed on the agarose gels, which were stained with ethidium bromide and photographed in the UV light.

RESULTS

It has been shown previously, that DNA in the left-handed state is resistant to methylation *in vivo* by M·*Eco*RI (33,34). Thus the *Eco*RI site located within the sequences capable of forming left-handed DNA in living *E.coli* cells show a slower kinetics of methylation relative to *Eco*RI sites located within the B-type sequences on the same molecule. These results were interpreted in terms of Z-DNA formation *in vivo* (33,34). Since little is known about the H-DNA formation in living cells, we used a parallel approach to compare the *in vivo* Dam methylation patterns of the plasmids listed in Fig. 1. All of the constructs used contain pur-pyr blocks. Only two of them, namely pPP1 and pPP2, were capable of forming intramolecular triplexes. Structural behavior of pPP1 insert was reported previously (19) and appeared to be very similar to the behavior of pPP2 insert as judged by DEPC modification studies (Fig. 2A). Briefly, both plasmids contain two $(AG)_7$ blocks which are necessary to form an intramolecular triplex structure *in vitro* in spite of the spacer sequences placed between them. The triplex formation can be observed at neutral pH if $-\sigma$ value is 0.06 and higher (Fig. 2A, lane 6). The decreased pH reduces the free energy derived from supercoiling

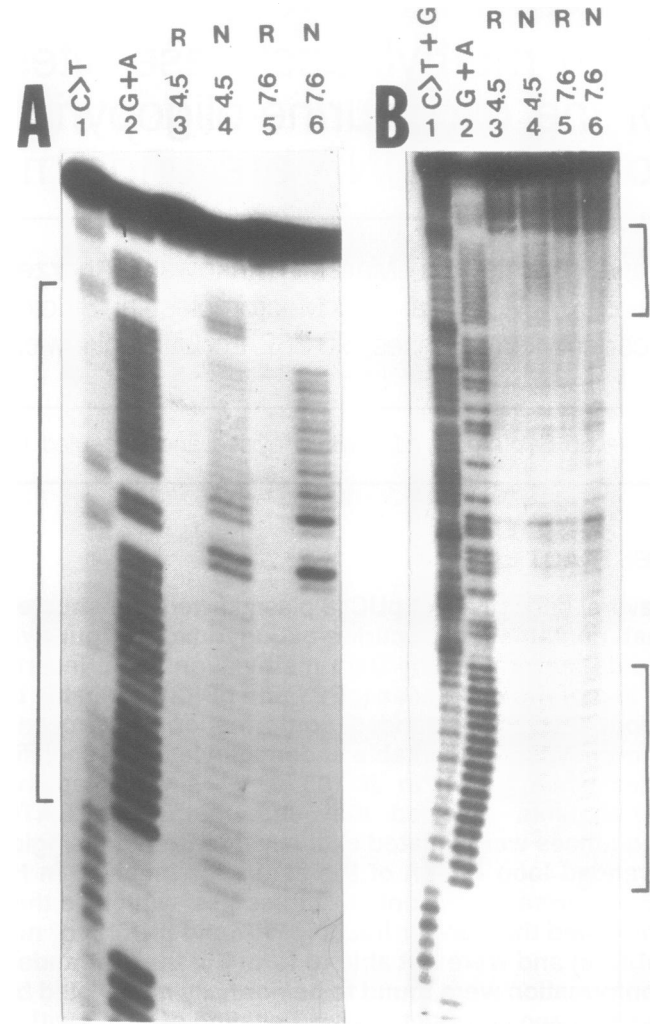

Fig.2. Sequencing of DEPC-modified pPP2 and pKK42 plasmids. Panel A: pPP2 plasmid suspended in Tris-HCl, pH 7.6 or acetate buffer, pH4.5 was modified with DEPC (19), cleaved with *Hind*III/*Sac*I, labelled by α^{32}PdATP and Klenow polymerase. The insert was recovered from a polyacrylamide gel and treated by piperidine followed by sequencing electrophoresis. R−relaxed plasmid, N−native plasmid. Sequencing reaction C>T and G+A are shown in lanes 1 and 2, respectively. The sequence of insert is indicated by brackets. Panel B: Results of DEPC modification obtained with pKK42. Brackets indicate the two $(AG)_7$ blocks. Other details as in Panel A.

necessary for the formation of the structure, which at pH 4.5 most likely represents the mixture of both isomeric forms (Fig. 2A, lane 4). It is important to note that pPP1 contains 3 Dam sites around the investigated sequence. Two of them are located within *Bam*HI recognition sequences which flank the pur-pyr block, whereas the third one is present in the spacer separating two $(AG)_7$ blocks (see Fig. 1). The plasmid contains also 14 other GATC sites located somewhere else on the vector molecule (36). pPP2, which is similar in the structural behavior to the insert to pPP1, differs only by having shorter spacer separating two $(AG)_7$ blocks as well as by having one more GATC site in the spacer (Fig. 1).

Two other plasmids, namely pPPK and pKK42, were used as controls. pPPK contains only one $(AG)_7$ block surrounded by two Dam sites. The pur-pyr $(AG)_7$ sequence is, however, too short to form the stable triplex by itself, since we failed to detect it by DEPC and OsO_4 both at neutral or acidic pH and at the

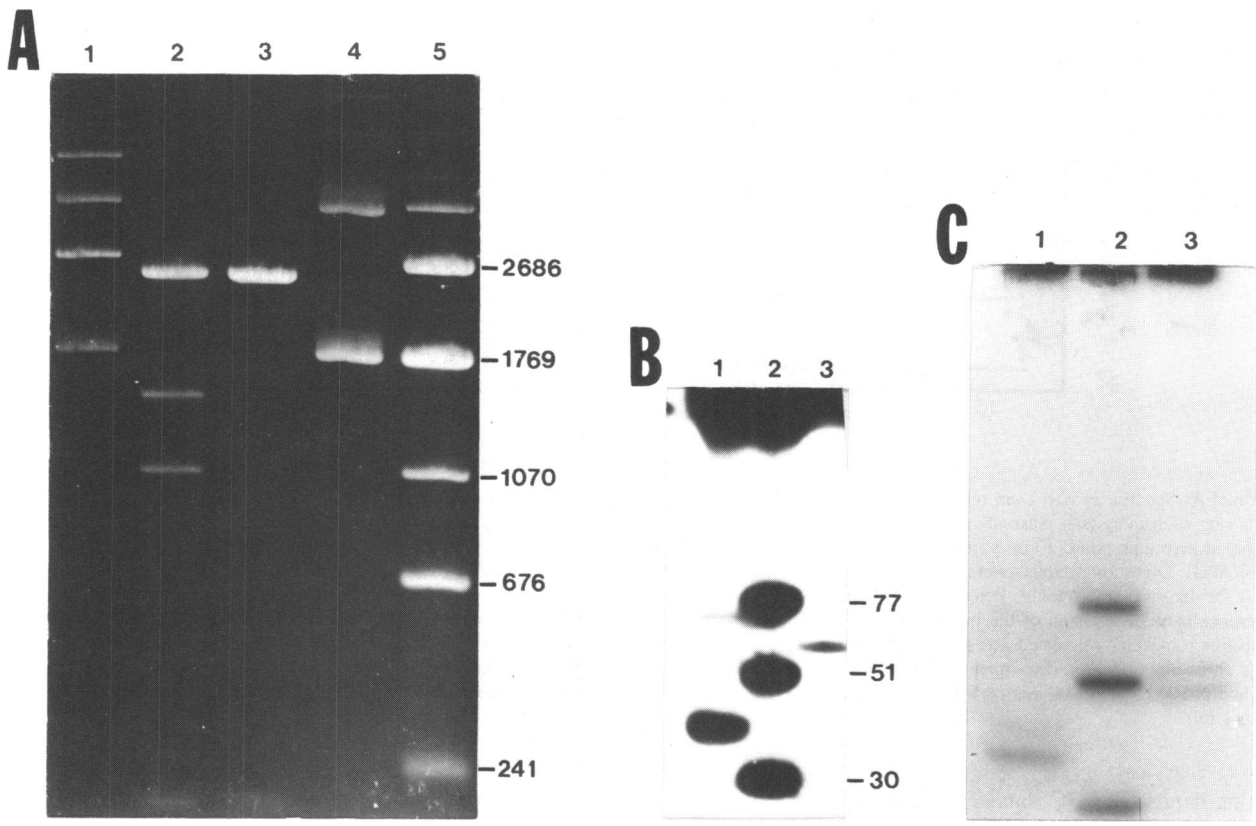

Fig. 3. Mapping of the *in vivo* Dam undermethylated sites on pPP1 and pPP2 plasmids. JM 105 *dam*$^+$ cells harboring the pPP1 or pPP2 were grown overnight on M9 media without chloramphenicol amplification. Plasmid DNA after isolation was purified by CsCl/ethidium bromide banding. Panel A: 1% agarose gel electrophoresis of the digestion products of pPP1 obtained with 100 fold excess of *Mbo*I (lane 1); *Mbo*I and *Ava*II (lane 2); *Ava*II (lane 3). Intact plasmid is shown in lane 4. Size markers are indicated in lane 5. Panel B: Autoradiogram of 10% polyacrylamide gel after electrophoresis of Klenow labelled digestion product of pPP1 obtained with *Eco*RI/*Mbo*I (lane 1) and *Hind*III/*Mbo*I (lane 3). Radioactive size markers of 30, 51 and 77 bp in length are shown in lane 2. Panel C: Same as Panel B except that pPP2 plasmid was used. Radioactive size markers shown in lane 2 are the same as in Panel B.

native $-\sigma$ value (not shown). Still another control molecule which is not able to form intramolecular triplexes is represented by pKK42. This plasmid contains a relatively long spacer sequence placed between two $(AG)_7$ blocks. Using this molecule no indication of triplex formation was detected based on the DEPC modification studies performed at native $-\sigma$ and neutral or acidic pH (Fig. 2B).

GATC sequences located between triplex forming $(AG)_7$ blocks are unmethylated *in vivo*

When pPP1 plasmid was grown and isolated from JM 105 (*dam*$^+$) without chloramphenicol amplification (Fig. 3A, lane 4) and than cleaved with 100 fold excess of *Mbo*I, partial linearization was achieved (Fig 3A, lane 1). Thus, some of GATC site(s) apparently were not completely methylated by Dam methyltransferase, since only unmethylated recognition sequences are sensitive to *Mbo*I cleavage. In similar and parallel experiment in which pUC18 vector DNA was grown and isolated in similar conditions, no sign of *Mbo*I sensitivity was detected. Thus, we could deduce that the incomplete Dam methylation site is most likely located within the GATC sequences of the insert of pPP1. Digestion of pPP1 with *Ava*II resulted in formation of two bands 2511 and 222 bp in length (Fig. 3A, lane 3) since two *Ava*II sites are present on the vector DNA (36). Electrophoresis of the products of double digestion with *Ava*II and *Mbo*I revealed two extra bands. Their length (1446 and 1065 bp) unambiguously indicates that the undermethylated Dam site(s) is present within the synthetic insert of pPP1. The results shown in Fig. 3A are in perfect agreement with another mapping experiment presented on Fig. 3B. There are three GATC sequences within the insert of pPP1. Thus, we addressed the question as to which one actually is partially methylated. When the plasmid DNA was cleaved with *Eco*RI/*Mbo*I and the digestion products, after labelling with α^{32}PdATP and Klenow polymerase, were separated on polyacrylamide gel, the detected radioactive band was 39 bp long. This corresponds to the distance between the *Eco*RI site (on the polylinker of the vector) and the GATC site located on the spacer sequence separating two $(AG)_7$ blocks (Fig 3B, lane 1). Within the labelled products obtained by digestion of pPP1 with *Hind*III and *Mbo*I a 59 bp long fragment appears (Fig. 3B, lane 3). The length of this fragment corresponds to the distance between *Hind*III site (on the polylinker of the vector) and GATC sequence of the spacer separating $(AG)_7$ blocks.

Results of the experiment indicating partial Dam methylation of two GATC sites present in between both $(AG)_7$ blocks on pPP2 molecule are shown on Fig. 3C. Plasmid isolated from JM105 host cells was cleaved with *Eco*RI/*Mbo*I or with *Hind*III/*Mbo*I and the products after labelling were electrophoresed on polyacrylamide gel. Within the *Mbo*I/*Eco*RI products two radioactive bands were detected: 39 and 43 bp in length, indicating that both GATC sites of the spacer separating $(AG)_7$ blocks were undermethylated (Fig. 3C, lane 1). *Hind*III/*Mbo*I cleavage of pPP2 resulted in formation of 49 and

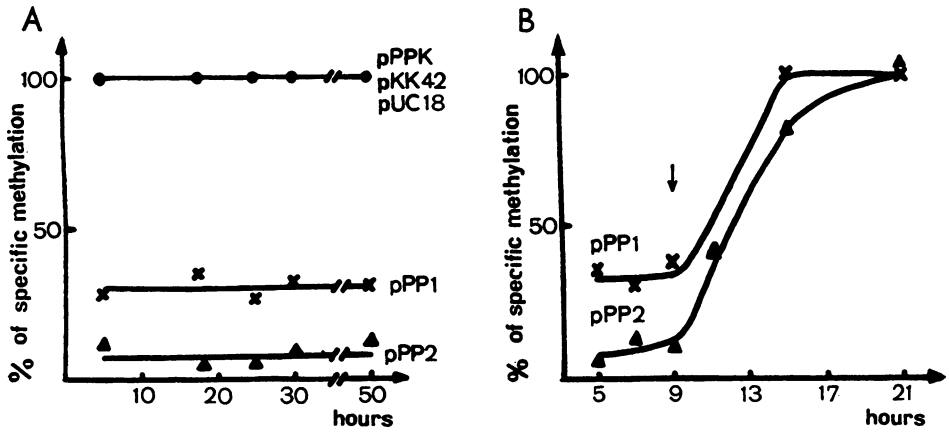

Fig. 4. Panel A: Specific *in vivo* Dam methylation of inserts of plasmids listed in Fig. 1. as a function of growth time in the cell cultures. 5 ml of JM105 *dam*$^+$ cells harboring each individual plasmid were grown for 7 hours at 37°C in M9 media (41) and 2.5 ml of cells suspension was used to inoculate 200 ml culture (M9 media) at zero time point. 10 ml portions of cell suspension were taken for plasmid isolation at the indicated time intervals and the isolated DNA was digested with *MboI*/*AvaII*. Digestion products were separated on 1% agarose gels and photographed under UV light after ethidium bromide staining. Negatives of the pictures were used for densitometric tracing. Percentage of the specific methylation within the GATC sequences of the inserts (plotted on the vertical axis) was calculated as a difference between the sum of the integrated *AvaII*/*AvaII* longer band and both *AvaII*/*MboI* bands (cf. Fig. 3A lane 2) assumed as 100% and the percent of *MboI* sensitive molecules calculated according to the formula: (*MboI*/*AvaII* + *MboI*/*AvaII*) · 100% · (*MboI*/*AvaII* + *MboI*/*AvaII* + *AvaII*/*AvaII*)$^{-1}$. Each point represents an average of three determinations. Panel B: Effect of chloramphenicol on the percentage of methylation of GATC sites of the triplex forming inserts of pPP1 and pPP2. Experiment was performed as described for Panel A. Arrow indicate the time point at which chloramphenicol was added to the cell cultures (170 μg per ml).

53 bp long bands (Fig. 3C, lane 3), again indicating that GATC sequences of the spacer of pPP2 are undermethylated *in vivo*.

On the basis of the results shown in Fig. 3A and 3B we can conclude that one out of 17 GATC sequences present on the pPP1 plasmid or two out of 18 GATC sites on pPP2 are undermethylated *in vivo*. Thus, the observed effect is extremely specific. The undermethylated sites are located within the single-stranded loop of the triplex detected previously (19) and in present studies *in vitro* (Fig. 2).

An effect of the specific Dam undermethylation *in vivo* is restricted to the sequences capable of forming intramolecular triplexes *in vitro*

It was of interest to compare the Dam methylation patterns of 5 plasmids listed in Fig. 1 grown in the JM 105 (*dam*$^+$) as a function of time. Sensitivity to *MboI* cleavage was used as a probe (Fig. 4A). Thus, JM 105 cells harboring each individual plasmid were grown in M9 media. From aliquots taken from the growing culture at the indicated time intervals plasmids were preparated (39), digested with *AvaII* and *MboI*, and then separated on agarose gels. Densitometric trace of the negative pictures of the gels permitted us to calculate the ratio between the intensities of the fully methylated plasmid molecules and the portion of the molecules which were specifically cleaved by *MboI* (therefore, specifically undermethylated *in vivo*) within the synthetic insert. We found that pPPK, pKK42 and pUC18 plasmids were totally insensitive to *MboI* restrictase as isolated in the course of growth because of full methylation. It is important to remember that inserts of pPPK and pKK42 do contain pur-pyr motifs. However, we failed to detect triple-stranded DNA within this molecules by widely used chemical probes such as DEPC or OsO_4 (12–15,18,19) at a native superhelix density and pH 7.6 or 4.5 (not shown). Simply, $(AG)_7$ tract of pPPK seems to be too short to form the triplex by itself, whereas the random sequence of the spacer separating the $(AG)_7$ blocks of pKK42 provides an energy barrier which protects against intramolecular triple-stranded DNA formation between two pur-pyr sequences. Thus, all three plasmids, namely pPPK, pKK42 and pUC18 served as controls in our studies and provided the information that sequences not capable of forming intramolecular triplexes are fully methylated *in vivo*.

As expected, the pPP1 insert appears to be partially methylated *in vivo* at the GATC sequence located between two $(AG)_7$ blocks. Virtually no change in the percentage of the specific undermethylation was observed up to 50 hours of the growth of the culture. Very similar results were obtained with pPP2, for which the percentage of the specific undermethylation of the GATC located within the single-stranded loop of the triplex forming sequence was much higher relative to pPP1. This result is likely due to the presence of two Dam sites within the loop forming spacer. Our *in vitro* studies of pPP2 showed that its insert sequence behaves in a very similar way to that of pPP1 (19). Thus, the triple-stranded state can be shown by DEPC modification at pH 7.6 and superhelical density −0.06 (Fig. 2) and the acidic pH significantly lowers the energy necessary for its formation (not shown).

In summary, the specific undermethylation of GATC sites *in vivo* seems to be restricted to these sequences which are able to form triple-stranded DNA. The effect depends on the sequence environment in a very specific way. Sequences surrounding undermethylated sites in pPP1 and pPP2 are different since the spacers between $(AG)_7$ blocks are not identical. Both molecules, however, are partially methylated. Close proximity to one $(AG)_7$ stretch is not sufficient for partial methylation. This situation is provided in control molecules: pKK42 and pPPK which are quite efficiently modified by Dam methylase *in vivo*. What seems to be necessary is the appropriate proximity of two pur-pyr blocks between which the GATC sequence is placed. This proximity correlates well with the capacity of the pur-pyr stretches to assume H-form DNA. Thus, our results suggest that Dam methylation system is sensitive to the structure of DNA and that the specific,

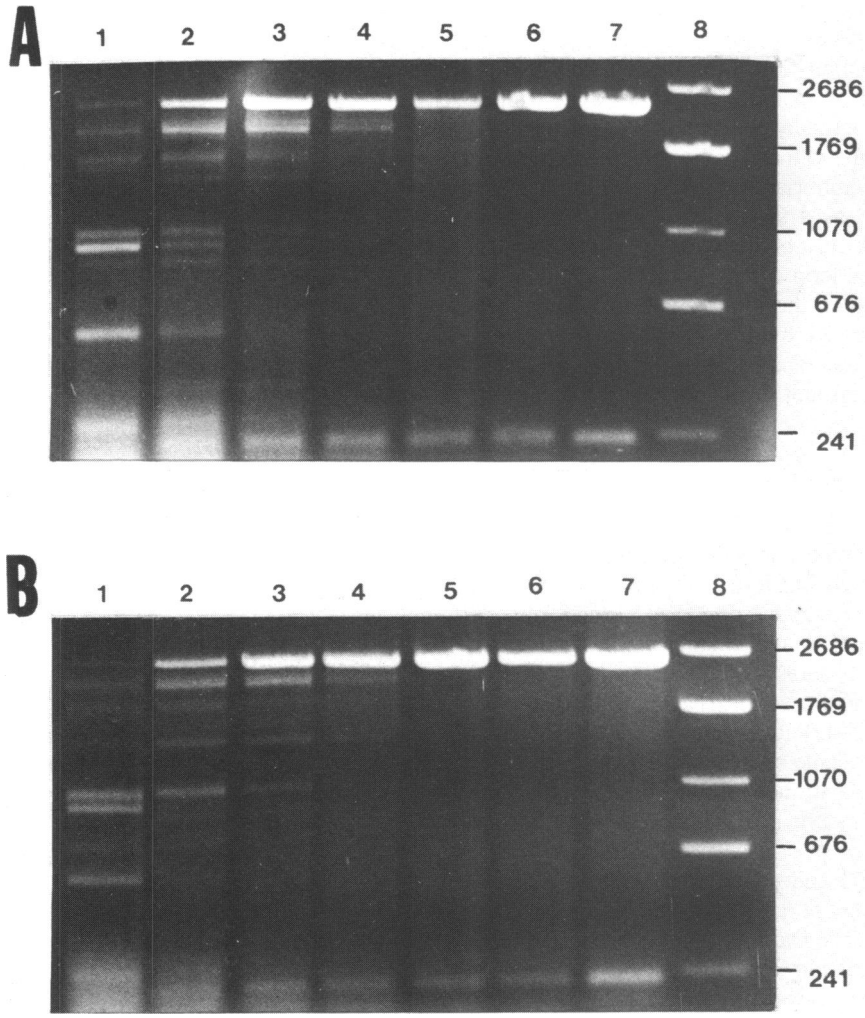

Fig. 5. *In vitro* methylation studies of pPPK (Panel A) and pPP1 (Panel B) obtained from GM2163 *dam*$^-$ *E.coli*. 2 μg of native DNA was methylated with 10 units of Dam methylase in the buffer containing 50 mM Tris-HCl, pH 7.6, 10 mM EDTA, 5 mM β-mercaptoethanol and 80 μM S-adenosylmethionine. Reaction was stopped after 2, 5, 7.5, 10, 15 and 20 minutes (lanes 1−6, respectively) by phenol treatment. DNA after extraction with ether (3 times) was precipitated, digested with *Mbo*I (100 fold excess) and *Ava*II and electrophoresed on 1% agarose gel. Lane 7 contains plasmid digested with *Ava*II. Size markers are shown in lane 8.

incomplete methylation described herein is due to the formation of the triple-stranded DNA *in vivo*.

Chloramphenicol (CM) amplification of pPP1 and pPP2 leads to the fully methylated molecules

We also tested the effect of chloramphenicol on the degree of methylation of pPP1 and pPP2 in JM 105 cells as a function of amplification time. As shown on Fig. 4B, the addition of chloramphenicol to the growing cultures resulted in complete methylation of the otherwise partially methylated GATC sites of the triplex loop. The complete methylation could be achieved within several hours after drug administration. CM is a widely used bacteriostatic agent that interferes with bacterial protein synthesis. Its effect on the Dam methylation of GATC sites within the triplex loop suggests that bacterial host cells contain a protein dependent mechanism for protecting triplex forming sequences against Dam methyltransferase action. The hypothesis suggested above is supported by the *in vitro* Dam methylation studies described below.

Kinetics of *in vitro* methylation of pPP1 and pPP2 inserts are different to those observed *in vivo*

Fig. 5 shows the results of the *in vitro* methylation studies of pPPK (Fig. 5A) and pPP1 (Fig. 5B). Native plasmids were grown in *E.coli* GM2163 (*dam*$^-$) strain (37) and the DNA after isolation was methylated with Dam methylase as a function of time. The methylated samples were digested with *Mbo*I and *Ava*II and electrophoresed on agarose gel. As can be seen, the control molecule (pPPK) becomes steadily and increasingly resistant to *Mbo*I digestion. After 15 minutes of exposure to Dam methylase the plasmid becomes completely resistant to *Mbo*I. The same results were obtained for the pPP1 plasmid. After 15 minutes of reaction, the plasmid was completely methylated including the Dam sites within the triplex loop sequence as evident by the absence of 1446 and 1065 bp long bands. We also performed experiment similar to that shown in Fig. 5B using the pPP1 plasmid at mean $-\sigma = 0.08$. No substantial inhibition of methylation of the Dam site within the insert (as compared to other sites) was detected. Similar results were obtained with pPP2 (not shown).

Our *in vitro* methylation studies indicate that the rate of methylation of GATC sequences within the inserts of pPP1 and pPP2 do not differ substantially from the methylation rates of other Dam sites of the vector, in spite of the preformed triplex. We interpret this result in terms of the dynamic character of H-DNA which is in equilibrium with B-form in solution. Moreover, the enzyme may shift the equilibrium toward duplex DNA which is than efficiently utilized as a substrate by Dam methylase. Clearly some other factors must be involved *in vivo* that protect the Dam sites of the triplex loop against complete methylation. A striking difference between the *in vitro* and *in vivo* kinetics of methylation is represented by the fact, that *in vivo* the percentage of undermethylated Dam sites of the pPP1 and pPP2 inserts remains virtually constant throughout the entire growth time tested (Fig. 3A).

DISCUSSION

Non-B DNA structures may play important roles in number of cellular processes (reviewed in Refs. 32 and 42). Several conformations such as left-handed Z-DNA, cruciforms or triplexes are well documented and characterized by *in vitro* studies. Recently, genetic systems were developed and applied to demonstrate the existence of cruciforms and left-handed DNA in *Escherichia coli* (33–35,42). In similar approach, which is presented in this paper, we have shown that pur-pyr sequences capable of adopting a triple-stranded DNA *in vitro* effect the cellular *dam* methylation function of *E.coli* cells *in vivo*. For the purpose of our studies several plasmids were constructed (Fig.1). Two of them, namely pPP1 and pPP2, were able of adopting H-form DNA under the stress of negative supercoiling at neutral and acidic pH (19) (Fig.2). The Dam recognition sequences were placed both outside the pur-pyr blocks (necessary for triple-stranded structure formation) as well as between them i.e. within the sequence of the loop region. Control molecules were similar in the sense of location of Dam sites but the potential of forming H-DNA was abolished either by extending the loop size sequence (pKK42) or by deleting one of the two $(AG)_7$ blocks (pPPK).

We found that GATC sequences located within the loop region of triple-stranded DNA forming inserts of the supercoiled plasmids are undermethylated *in vivo* by *dam* methyltransferase gene product. In order to achieve the protection from Dam methylase action *in vivo* the potential to form H-DNA by pur-pyr stretch seems to be necessary. Control molecules unable to adopt triple-stranded conformation *in vitro* were found to be fully methylated *in vivo*. Thus, we conclude that the specific undermethylation state may reflect the triple-stranded DNA formation in living cells. H-DNA *per se* is not sufficient, however, since *in vitro* studies have demonstrated that the methylation rate of GATC sites located within the triplex loop does not differ substantially from those of other Dam sites located elsewhere on the vector sequence. An additional factor or factors must operate in the living *E.coli* cells that protect the GATC sequences of the loop of H-DNA against Dam methylase activity. The nature of these factors is uncertain, although they would appear to be proteins since complete methylation can be readily induced *in vivo* by chloramphenicol administration to the cell cultures.

Dam methyltransferase is an important procaryotic protein involved in regulation of several biological processes such as DNA mismach-repair, gene expression, transposition, replication and chromosome segregation (for review see Ref. 40). We believe that results presented in this paper may offer an intriguing model for studying the DNA structures related to the regulatory processes mediated by Dam methylation *in vivo*.

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