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Sequence Specificity of DNA Adenine Methylase in the Protozoan *Tetrahymena thermophila*

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The sequence specificity of the *Tetrahymena* DNA-adenine methylase was determined by nearest-neighbor analyses of in vivo and in vitro methylated DNA. In vivo all four common bases were found to the 5′ side of N^6-methyladenine, but only thymidine was 3′. Homologous DNA already methylated in vivo and heterologous *Micrococcus luteus* DNA were methylated in vitro by a partially purified DNA-adenine methylase activity isolated from *Tetrahymena* macronuclei. The in vitro-methylated sequence differed from the in vivo sequence in that both thymidine and cytosine were 3′ nearest neighbors of N^6-methyladenine.

The DNA of eucaryotes and procaryotes contains one or more species of methylated base: viz., 5-methylcytosine and N^6-methyladenine (MeAde). Although these species have been the subject of much investigation and discussion (5a, 9), their function in eucaryotes remains to be elucidated. In this regard, the ciliate *Tetrahymena thermophila* presents a convenient system for studying the role of DNA methylation in gene expression. The macro- and micronuclei are derived from the same zygotic nucleus at conjugation. However, in the vegetative cell the two nuclei differ dramatically in structure and function (1, 2): (i) the micronucleus serves as the germinal nucleus, divides mitotically, remains diploid, but synthesizes little or no RNA; and (ii) the macronucleus divides amitotically, is endoreplicated (contains 23 times more DNA than the micronucleus), and is transcriptionally active. In macronuclear DNA, approximately 0.8% of the adenine bases is modified to MeAde, whereas micronuclear DNA contains no detectable MeAde (3, 7); both DNAs are devoid of 5-methylcytosine (7, 10). Finally, MeAde residues are preferentially located in internucleosomal linker DNA sequences (10). Because of this nonrandom distribution of methylated bases, we considered it important to investigate what factors contribute to this pattern. Our first approach to this question was to elucidate the actual nucleotide sequence(s) methylated.

To determine the specificity of DNA methylation in vivo, we took advantage of the fact that MeAde is the only DNA base labeled when *Tetrahymena* is grown in medium containing L-[*methyl*-^{3}H]methionine (7, 10). Thus, in vivo-labeled DNA can be analyzed in a manner similar to that used for studying the in vitro specificity of DNA methylases (5, 6, 8). In brief, the labeled DNA was enzymatically degraded to mono- and dinucleotides that were separated by ion-exchange chromatography. The labeled MeAde (A*)-containing dinucleotides were purified, fractionated according to base composition, and sequenced. We observed that all four standard bases were 5′ nearest neighbors of A* (Table 1); in contrast, thymidine was the only 3′ nearest neighbor. This result was obtained with several independently labeled macronuclear DNA preparations. If we assume that methylation is by a single enzyme species, the simplest sequence recognized would be 5′...N-A-T...3′. If this DNA sequence alone were sufficient for methylation, then 38% of the adenine residues in *Tetrahymena* DNA should be methylated. However, only 0.8% of the adenine residues is MeAde (3, 7, 10).

To study sequence specificity further, we decided to isolate the *Tetrahymena* DNA methylase and study its specificity with two acceptor DNA substrates: viz., *Micrococcus luteus* DNA (72% G+C) and *Tetrahymena* macronuclear DNA (24% G+C). The DNA methylase activity was isolated from purified macronuclei as follows. *T. thermophila* strain B-1868-VII was grown at 28°C in enriched proteose peptone. Macronuclei were isolated essentially as described previously (4) from 20 liters of late-log-phase cells and frozen. After being thawed at 0°C, the macronuclei were washed in cold microsomal medium (0.25 M sucrose, 0.10 M KCl, 0.006 M $MgCl_2$, 0.001 M dithiothreitol, 0.01% [wt/vol] spermidine-HCl, 0.01 M Tris-hydrochloride [pH 7.2]) and resuspended with 5 ml of M-buffer (0.05 M Tris-hydrochloride [pH 7.8], 0.001 M dithiothreitol, 0.001 M disodium EDTA,

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TABLE 1. Analysis of nearest neighbors to A* after in vivo and in vitro DNA methylation

Dinucleotide	Percentage of ^3H label found in dinucleotides from:		
	Tetrahymena DNA		Micrococcal DNA
	In vivo methylation	In vitro methylation	In vitro methylation
C-A*	17.5	2.3	18.4
G-A*	12.7	3.5	10.5
A-A*	20.9	18.1	6.9
T-A*	12.7	27.9	4.3
A*-C	0.2	33.7	57.6
A*-G	0.1	0.9	0.8
A*-A	0	1.3	0.6
A*-T	36.1	12.3	1.1

10% [vol/vol] glycerol). An equal volume of M-buffer containing 0.8 M NaCl was added slowly during stirring. The nuclei were further lysed by sonication and diluted with 3 volumes of M-buffer. After clarification by centrifugation (30 min at 17,000 × *g*), 16 μl of 0.8% (wt/vol) protamine sulfate was added for each unit of absorbancy at 260 nm (A_{260}) in the supernatant. After being stirred in the cold for 10 min, the mixture was again clarified by centrifugation (10 min at 17,000 × *g*). The supernatant was adjusted to 65% saturation in ammonium sulfate and stirred slowly for 20 min at 4°C. After centrifugation (20 min at 20,000 × *g*) the precipitate was dissolved in and dialyzed against M-buffer containing 0.04 M NaCl (MN-buffer). The dialysate was applied to a DEAE-cellulose column (8-ml bed volume), equilibrated in MN-buffer, and washed with the same buffer.

Under these conditions, DNA adenine methylase activity passes through the resin along with about 25% of the input A_{280} units. The peak fractions of enzyme activity were pooled and concentrated by precipitation in ammonium sulfate as described above. The precipitate was suspended and dialyzed against MN-buffer containing 50% (vol/vol) glycerol. Enzyme activity in this fraction was not lost after several months storage at −20°C. Because the activity was isolated from purified macronuclei and because the crude fractions contained other methyl acceptors, we cannot calculate the extent of purification. DNA methylase activity was assayed (as in Fig. 1) in 0.15 ml (total volume) for 20 min at 37°C and terminated by the addition of 2 ml of 5% (wt/vol) cold trichloroacetic acid containing 4 μM unlabeled *S*-adenosylmethionine. After 20 min at 4°C the acid-precipitable fraction was collected by filtration. Preparation and isolation of in vivo-labeled DNA were as described previously (10).

Tetrahymena DNA was methylated to a high-

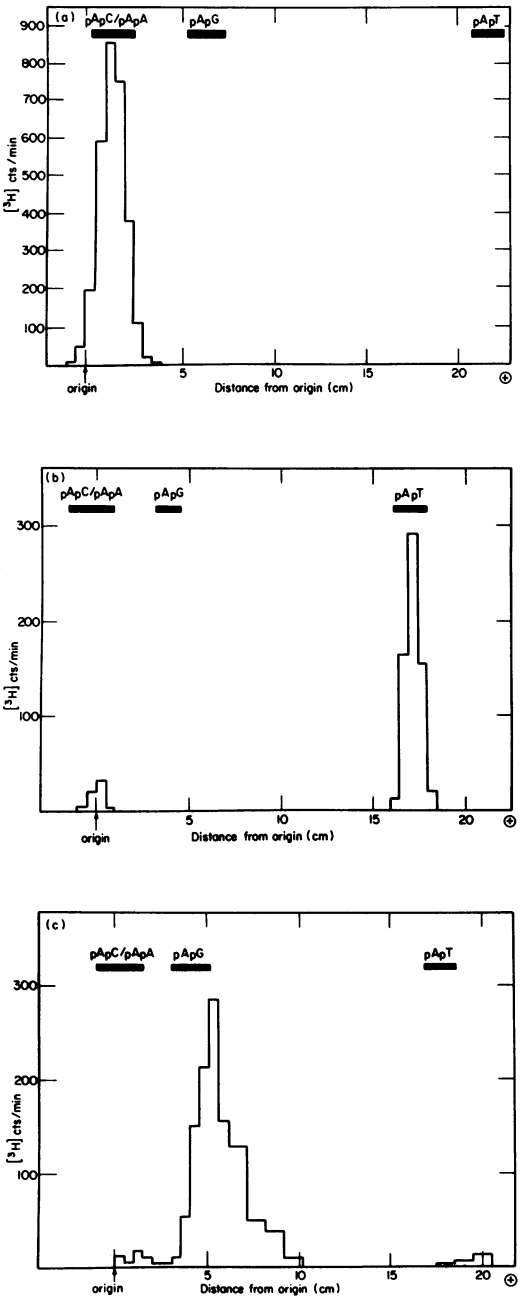

FIG. 1. Electrophoretic analysis of [^3H]A* dinucleotide fractions obtained by DEAE-cellulose chromatography (see Fig. 2). Peaks II, III, and IV were pooled separately and desalted on a 1-ml DEAE-cellulose column (5, 6, 8). Samples were subjected to paper electrophoresis (Whatman 3MM) at pH 1.9 for 3.5 h at 2 kV on a Savant flat-plate electrophoresis apparatus. Authentic adenine-containing deoxydinucleotides of known sequence were included as reference markers (indicated by solid bars). Analysis of peaks II, III, and IV are shown in a, b, and c, respectively.

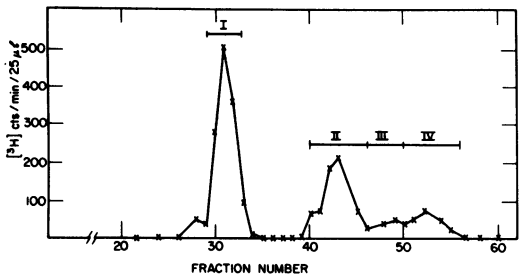

FIG. 2. Separation of [^{3}H]A*-labeled mono- and dinucleotides from *M. luteus* DNA methylated in vitro by *T. thermophila* DNA methylase. In vitro methylation was in 220 nM *S*-adenosyl-L-[*methyl*-^{3}H]methionine (3.3 Ci/ml), 33 mM Tris-hydrochloride (pH 7.5), 3.3 mM disodium EDTA, 4.3 mM 2-mercaptoethanol, 70 to 100 µg of DNA per ml, and 160 to 660 µg of enzyme fraction protein per ml. After incubation for 7 h at 37°C, Sarkosyl NL97 (Geigy Chemical Corp.) was added to 2% (wt/vol) final concentration, and the DNA was purified and extensively dialyzed against H_2O. *M. luteus* DNA was from Sigma Chemical Co. The conditions for enzymatic digestion and chromatography on DEAE-cellulose (7 M urea) were as previously described (5, 6, 8).

er specific activity than was *M. luteus* DNA (data not shown), although *Tetrahymena* macronuclear DNA is already methylated in vivo (0.8% of the adenines is MeAde), whereas *M. luteus* DNA has no MeAde (<1 MeAde per 10,000 adenine residues; D. Swinton and S. Hattman, unpublished data). It is a common feature of higher eucaryote nuclear DNA that it can be further methylated in vitro by its own DNA methylase. The percentages of dinucleotide species were calculated from electrophoresis at pH 1.9 (Fig. 2) and pH 3.5 (not shown) and from the proportions of sequence isomers obtained in the nearest-neighbor analysis. In vitro methylation differed in sequence specificity from that observed in vivo. For example, when *M. luteus* DNA was methylated in vitro, the 3′ nearest neighbor was almost exclusively cytosine (Table 1). Also, the frequency of G-A* + C-A* versus A-A* + T-A* was threefold higher. This may be because of the threefold higher G + C content of *M. luteus* DNA. However, when *Tetrahymena* macronuclear DNA was methylated in vitro and analyzed, the proportions of A*-containing dinucleotides were significantly different from methylation of *M. luteus* DNA. For example, (A*,T) and (A*,A) were present in much higher relative proportions, and (A*-C) plus (A*-G) was reduced more than twofold. These data, including the sequence(s) and frequency of each dinucleotide, are summarized in Table 1 (column 2). All four bases are found as 5′ nearest neighbors; however, adenine and thymidine are present nine times more frequently than are cytosine and guanine. Although not strictly correlated with base composition, these results suggest that the 5′ position is degenerate. Furthermore, both cytosine and thymidine are present as 3′ nearest neighbors, and even though *Tetrahymena* DNA has a low cytosine-to-thymidine ratio, there was more A*-C than A*-T. The results of these studies indicate that in vitro DNA methylation by the *Tetrahymena* enzyme produces N-A* and A*-Py (where Py is preferentially C). It is important to keep in mind that *Tetrahymena* macronuclear DNA is already methylated in vivo. Therefore, in vitro methylation labels MeAde residues at those sites which were not methylated in vivo. It is not clear whether this is responsible in any way for the difference observed in sequence specificity for in vivo versus in vitro methylation. Several explanations are possible: (i) there is a single methylase enzyme whose specificity is altered during purification or when it is acting on DNA versus chromatin; (ii) there may be two enzymes present in vegetative cells, but only one is functional in vivo; (iii) the methylation pattern is actually the same in vivo and in vitro, but an endonuclease specifically cleaves all A*-C phosphodiester bonds in vivo; and (iv) the A-C containing recognition sequences are inaccessible in vivo to DNA methylase, but after deproteinization these sites are accessible to the methylase in vitro. This hypothesis implies there is specific compartmentalization of potential methylation sequences in vivo (presumably through sequence-specific protein-DNA interactions). This notion is particularly attractive in light of several recent findings: viz., MeAde is preferentially located in internucleosomal linker DNA (10), and nucleosome core DNA sequences are a subset of the total *Tetrahymena* genomic complexity (K. Pratt and S. Hattman, submitted for publication). Finally, it is also possible that N-A-T is only a portion of the methylase recognition site; i.e., the actual sequence may be four, five, or six (or more) bases, with adenine being the 5′ terminal position. Alternatively, N-A-T may be contained within a longer sequence, 5′...X-N-A-T-Y...3′, and only the base 5′ to adenine is degenerate. Whatever the case, it seems clear that N-A-T is not sufficient information to serve as a methylation site; this was confirmed in other experiments with restriction nucleases in which it was shown that G-A-T-C sequences are not methylated (K. Pratt, Ph.D. thesis, University of Rochester, Rochester, N.Y. 1981). The resolution of these questions remains open for future investigation.

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