NUCLEIC ACID METHYLATION

Organizers: Gary Clawson, Dawn Willis, Arthur Weissbach and Peter Jones

March 31-April 7, 1989

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Nucleic Acid Methylation

Structure, Genesis and Function of mRNA Caps

CH 001 CAP ADDITION BY TRANS-SPLICING IN TRYPANOSOMES, John C. Boothroyd, MiMi P. Hsu and Michael L. Muhich, Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA 94306.

Trypanosoma brucei is a pathogenic protozoan which cause significant animal and human disease in Africa. They are the best studied genus within the order Kinetoplastida which includes such other genera as Leishmania and Crithidia. All of these genera use a process known as "trans-splicing" to process their mRNAs. This involves the splicing together of two discretely synthesized RNA molecules. The actual process appears highly similar to that involved in the well-studied cis-splicing of nuclear transcripts in higher eukaryotes. The resulting chimeric mRNA is comprised of a short, capped sequence of 39 nucleotides at the 5'-end followed by the protein-coding sequence. Trans-splicing is apparently involved in the synthesis of most, probably all nuclear-encoded mRNAs in these organisms. The 5'-region is termed the mini-exon or spliced-leader sequence and is orginally synthesized as a short precursor RNA of about 140 nucleotides, the 5'-end of which comprises the mini-exon sequence. This RNA is capped by a conventional 7-methyl-G in 5'-5' triphosphate linkage [Freistadt et al. (1987), N.A.R. 15:9861; Perry et al. (1987) PNAS 84:8190; Sutton and Boothroyd (1988) Mol. Cell Biol. 8:494]. However, it is unusual in apparently being a "cap-4" as the first 4 positions are modified. The precise structure of all the modifications is not yet known.

I will present data arguing for a role of trans-splicing in processing of polycistronic transcripts into monocistronic, capped mRNAs. Preliminary attempts at precise structural determinations are in progress.

DNA Methylation in Fungi and Plants

CH 002 BIOSYNTHESIS AND DISTRIBUTION OF METHYLCYTOSINE IN WHEAT DNA AND OTHER PLANTS. Hartmut Follmann, Hans-Jörg Balzer, and Roland Schleicher Fachbereich Chemie der Philipps-Universität, D-3550 Marburg (Germany)*

The amount of 5-methylcytosine in plant DNA is so much higher than in the DNA of all other eukaryotic organisms (up to 10 *vs.* 1 mol%) that one must assume fundamental differences in the functions, distribution within the genome, or biosynthesis of the modified base in the plant kingdom. We approach this question by characterizing the DNA methyltransferases present in selected plant species and tissues (*e.g.*, wheat, maize, and soy bean) and their specificity for DNA substrates of different complexity and methylcytosine content. Wheat (*Triticum aestivum*) embryos have proved the best source for isolation of highly active plant DNA methylase. This enzyme (Theiss, Schleicher & Follmann, Eur.J.Biochem.167, 89-96 (1987)) is indeed totally different from mammalian DNA methylases in size, specificity, and immunological properties. The enzyme is most active towards (plant) DNA substrates of an intermediate degree of methylation. Methylation of wheat DNA *in vivo* and *in vitro* is also distinguished from DNA methylation in mammalian cells by its very low sensitivity to 5-azacytidine inhibition. A substantial decrease in the methylcytosine content of wheat during the first 25 hours of germination (from 5.5 to 3.9 mol%) is, paradoxically, accompanied by an increase in enzyme activity. Although we have failed so far to detect more than one DNA methylase in plants it is likely that one has to differentiate bulk DNA methylation (of unknown function) from the specific methylation of a small number of C residues in single copy genes (engaged in the regulation of gene activity). Our current efforts are therefore centered on an analysis of the methylation status of individual wheat genes (kindly provided by Drs.D.Bartels, P.T.H.Brown, and C.Fritz, Max-Planck-Institut für Züchtungsforschung, Köln) at different stages of development of the wheat embryo.

Supported by a grant from Deutsche Forschungsgemeinschaft (Fo 50/14) to H.F.

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CH 003 VIRUSES INFECTING A EUKARYOTIC GREEN ALGA ARE A NEW SOURCE OF DNA METHYLTRANSFERASES AND DNA SITE-SPECIFIC ENDONUCLEASES. Van Etten, J.L., Xia, Y., and Burbank, D.E. Department of Plant Pathology, University of Nebraska, Lincoln, NE 68583. We have isolated and partially characterized several large, complex, dsDNA containing (ca. 330 kbp), plaque forming viruses (NC64A viruses) which infect the unicellular, eukaryotic, Chlorella-like green alga strain NC64A. These viruses can be distinguished on the basis of plaque size, reaction with antibody, and the nature and abundance of methylated bases in their genomic DNAs. The concentration of methylated bases varies from viruses containing no 6-methyladenine (6mA) and 0.1% 5-methylcytosine (5mC) to one virus with 37% 6mA and 45% 5mC. Even though the host nuclear DNA contains 21% 5mC and 0.6% 6mA, at least some of the methylated bases in the viruses reside in different DNA sequences than those in the host. This result led to the finding that virus infection of the host results in the synthesis of DNA methyltransferases and DNA site-specific (restriction) endonucleases. Five different site-specific endonucleases have been identified so far. For example, virus NC-1A infected Chlorella NC64A cells contain an endonuclease, named CviBI, which recognizes the sequence GANTC and cleaves between G and A; CviBI does not cleave G^{m}ANTC sequences. NC-1A infected Chlorella cells contain the cognate methyltransferase, M.CviBI, which specifically methylates adenine in GANTC sequences and at least two other distinct methyltransferases; M.CviBII and M.CviBIII methylate adenine in GATC and TCGA sequences, respectively. The M.CviBIII gene was cloned, sequenced and a single open reading frame of 1131 bp identified. A comparison of the predicted amino acid sequence of M.CviBIII with M.TaqI, a bacterial isoschizomer, revealed 39% identity.

A second family of viruses that infect another strain of Chlorella (Pbi) have recently been isolated. Like the NC64A viruses, the Pbi viruses contain large dsDNAs genomes with various levels of methylated bases. Infection of Chlorella Pbi with the Pbi viruses also results in the synthesis of DNA methyltransferases and DNA site-specific endonucleases. Thus these Chlorella viruses are a new source of both adenine and cytosine methyltransferases and DNA site-specific endonucleases.

DNA Methylation in Prokaryotes

CH 004 THE DAM DNA-METHYLTRANSFERASES OF E. COLI AND ITS PHAGES T2 AND T4; Zoe Miner, Samuel Schlagman and Stanley Hattman, Department of Biology, University of Rochester, Rochester, NY 14627.

DNA (adenine-N^{6}) methyltransferases (MTases) recognizing the palindromic tetranucleotide sequence, 5'-GATC-3', are encoded by (dam) genes of various bacteriophages (e.g. T2, T4, T1, P1) and bacteria (e.g. Escherichia, Salmonella, Neisseria, Streptococcus). Cloning and nucleotide sequence analysis has revealed that these Dam polypeptides contain several regions of considerable amino acid sequence homology; and one of these regions (IV) contains a sequence motif, (Asp/Asn)-Pro-Pro-(Phe/Tyr), also found in MTases that methylate adenine in sequences other than GATC. The conservation of amino acid sequences among these enzymes suggests that they are in domains important for MTase function and specificity; i.e. substrate (S-adenosylmethionine, AdoMet) and DNA-nucleotide sequence recognition/interaction.

We have been focusing on the Dam MTases of phages T2 and T4, particularly because mutants (dam^{h}) are known that methylate DNA to higher extents than the enzyme from the wild-type (dam^{+}) parent. In addition, second site mutants (dam^{h} dam-x) have been isolated that abolish all DNA methylation ability. The normal substrate for the phage DNA MTases is DNA containing 5-hydroxymethylcytosine (hmC), because the viruses contain this base in place of C; however, C-DNA is still a good substrate for these enzymes. The two wild-type phage MTases differ in that the T2 enzyme adds about twice as many methyl groups per unit DNA than does the T4 enzyme.

A comparison of the wild-type T2 and T4 Dam^{+} encoded polypeptides revealed three amino acid differences; viz., at positions 20, 26 and 188. The latter is a conserved change (Asp → Glu) and does not appear to be involved in sequence specificity. The other two changes are located in homology region I, implicating this domain in nucleotide sequence recognition. Current efforts to prove this include production of chimeric enzymes and site-directed mutagenesis.

We have shown that the dam^{+} → dam^{h} mutation produces a Pro → Ser change at amino acid residue 126. A Phe →Val change at residue 127 prevents the MTase from methylating hmC - DNA, but not C-DNA. These two residues are contained in homology region III, also implicating this domain in nucleotide sequence recognition.

CH 005 STUDIES ON DNA METHYLATION AND RESTRICTION PROCESSES BY USE OF BACTERIAL VIRUS T7,

D.H.Kruger[a], T.A.Bickle[b], M.Reuter[a], C.D.Pein[c] and C.Schroeder[a],

Institute of Medical Virology[a] and Department of Chemistry[c], Humboldt University, DDR-1040 Berlin, G.D.R.; and Biocenter, University of Basel, CH-4056 Basel, Switzerland[b].

DNA modification-restriction enzymes of Escherichia coli cells can be grouped into 3 families, called type I, II, and III (1). - The ocr+ gene of T7 encodes the first known inhibitor protein specifically blocking a group of methylases and endonucleases which, in particular, belong to the type I (EcoB, EcoK) enzymes (2;3). - The appearance of recognition sites for type II enzymes (e.g., EcoRII, methylases EcoDam and EcoDcm) is strongly counterselected in the T7 genome (4), furthermore, there are additional mechanisms preventing the enzymes from acting on the remaining sites (5). For instance, EcoRII is a restriction enzyme which requires the coordinated presence of at least 2 recognition sites in the substrate DNA for its activity (6). Sites which do not fulfill this requirement are refractory to EcoRII but can be cleaved by this enzyme in the presence of a second, *susceptible DNA species (6) or oligonucleotides (CDP et al, submitted).* - The resistance of T7 DNA towards the type III enzyme EcoP15 is explained by the absolute "strand bias" of the non-symmetric sites (in which only one strand can be methylated) in the DNA molecule (4; CS et al, in prep). These data allow some understanding of how the endogenous DNA in EcoP15-encoding cells could be protected against "self-restriction" during replication. - The frequency and polarity of recognition sites in the DNA molecule may play a critical role in the function of certain restriction endonucleases and methylases.

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Structure of Methylated DNA and Chromatin

CH 006 *METHYLATED DNA-BINDING PROTEIN FROM MAMMALIAN CELLS*, Melanie Ehrlich, Xian-Yang Zhang, Prakash C. Supakar, Rana Khan, and Kenneth C. Ehrlich[1]. Tulane Medical School, New Orleans, LA 70112 and the [1]Southern Regional Research Center, U.S.D.A., New Orleans, LA 70112. Methylated DNA-binding protein (MDBP) binds to certain CpG-containing DNA sequences only when they are methylated at their C residues (1,2). In addition, other sites are recognized without DNA methylation but only if they contain TpG or TpA dinucleotides replacing the MpG (M, 5-methylcytosine) sequences of methylation-dependent MDBP sites. A 14 base-pair consensus sequence has been deduced from more than fifteen MDBP sites including prokaryotic sites, sites in anonymous DNA sequences cloned from MboI-digested human DNA, and sites in or near known genes of humans, rodents, herpes simplex virus type 1, or human cytomegalovirus. The latter group of viral and mammalian sites were identified by computer searches and verified as MDBP sites by band shift assays. The consensus sequence for MDBP recognition is 5'-RYm^{5}YRYYAm^{5}YRGm^{5}YRAY-3' (R, A or G; Y, T or C; m^{5}Y, M or T). It has an unusually large amount of degeneracy despite the fact that single base-pair mutations often abolish or greatly decrease recognition of a given site by MDBP (3,4). Methylation-dependent *prokaryotic and eukaryotic* MDBP sites display intermediate affinities for MDBP when they are hemimethylated compared to when they are bifilarly methylated or unmethylated. This could lead to DNA replication-coordinated effects on cell function. MDBP appears to be a ubiquitous mammalian DNA-binding protein (5). Its common occurrence in mammalian cells and the fact that it can bind in a methylation-independent manner to some sites and in a methylation-dependent fashion to others suggests that it performs some constitutive functions as well as some differentiation-associated roles in mammalian cells. Evidence for a role of MDBP in the regulation of transcription will be discussed.

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CH 007 **INACTIVATION OF GENE EXPRESSION AND STIMULATION OF DNA METHYLATION BY ALKYLATING AGENTS.** Robert Ivarie, Iain K. Farrance, Todd E. Arnold, Iris S. Hall and Julie Morris, Department of Genetics,University of Georgia,Athens, GA 30602,

GH_3 cells are an established tumor cell line from rat anterior pituitary that express the genes for both prolactin (PRL) and growth hormone (GH). PRL-deficient variants have been induced at >10% frequency by EMS and reverted at >20% by 5-azacytidine, (1-3). Deficient lines express the GH gene but have undergone a >50-fold loss in PRL expression. The defect appears to reside at the level of initiation of PRL gene transcription because variant cells synthesize greatly reduced levels of nuclear PRL transcripts that are correctly processed to mature messages bearing normal 3' and 5' termini. Furthermore, deficient cells are fully competent to initiate transcription from a chimeric CAT gene driven by both proximal and distal elements of the PRL promoter. *In vitro* assays also indicate that PRL-deficient cells contain all the known transcription factors (common and tissue-specific) that bind to proximal and distal elements of the PRL promoter. These observations are consistent with the idea that EMS induced enzymatic methylation of the prolactin gene itself (***cis*** model), not a gene encoding a transactivating factor (***trans*** model) that silenced the gene in a heritable manner. Indeed, poly(dC-dG)-poly(dC-dG) harboring ~2% hemi-ethylated CpG sites via EMS treatment can accept methyls ~20-fold more efficiently than a mock-treated alternating dC-dG polymer. Methods have been worked out to synthesize N7-ethyl-dGTP and place N7-ethylguanines sites 5' and 3' to cytosine in GCG sites to test whether the N7 adduct is responsible for enzymatic stimulation on EMS-treated substrates. 1. Ivarie, R., Morris., J.A. and Martial, J.A. (1982) Mol. Cell. Biol. 2, 179; 2. Ivarie, R. and Morris, J.A. (1982) Proc. Natl. Acad. Sci. USA 79, 2967; 3. Ivarie, R. and Morris, J. (1983) DNA 2, 113; 4. Farrance, I.K. and R. Ivarie (1985) Proc. Natl. Acad. Sci. USA 82, 1045; 5. Ivarie, R. and J. Morris (1986) Mol. Cell. Biol. 6, 97; 6. Davis, R.E., Morris, J. and Ivarie (1987) Mol. Endocrinol. 1, 102.

Ribosome Structure and rRNA Methylation

CH 008 *ERM* METHYLASES IN STAPHYLOCOCCI AND THEIR MODE OF SPREAD. Donald T. Dubin[1], Lucia E. Tillotson[1], Leslie Huwyler[2], and Ellen Murphy[2]. Dept. of Molecular Genetics and Microbiology, UMDNJ-Robt. Wood Johnson Med. School, Piscataway, NJ 08854[1] and The Public Health Research Institute, New York, NY 10016[2].

Resistance to the macrolide, lincosamide and streptogramin B ("MLS") groups of antibiotics is mediated by the *erm* class of methylases, which render bacterial ribosomes resistant to these agents by converting a single 23S RNA A residue to m_2^6A. Three evolutionarily distinct *erm* subclasses occur in staphylococci, *ermA*, *-B*, and *-C*. In recent surveys of clinical isolates we have found MLS resistance to be common in both *S. aureus* and coagulase negative staphylococci ("CNS"); and the prevalent *erm* genes to be *A* and *C*.

ErmC was initially discovered as part of a 3.7 kb *S. aureus* plasmid, pE194. Its current replicon in our area, both for *S. aureus* and CNS, has been found to be an unrelated 2.4 kb plasmid, pNE131, initially discovered in *S. epidermidis*. This replicon has apparently enjoyed extensive interspecific spread, presumably *via* mobilization by conjugative plasmids, or by transduction.

ErmA occurs in a transposon, Tn*554*, with high specificity for a single chromosomal *att* site, *att554*, in standard laboratory *S. aureus* strains. We have observed a diversity of 2° *att* sites in clinical isolates of *S. aureus* and CNS. One of these, "*att137*," was found to be the same as a site into which Tn*554* can be forced by transduction into a recipient deleted for *att554*. Comparison of junctional sequences has revealed a novel mode of information transfer associated with transposition: the 5'-adjacent chromosomal 6/7 residues of parent transposons become the 3' residues of daughters. Analysis of a current outbreak of methicillin resistant ("Mc^r") *S. aureus* has shown that all outbreak isolates are MLS^r due to *ermA*; and all contain Tn*554* in yet a different 2° site, "*att155*". Junctional sequences of inserts in this site were the same as those of a similar insert prevalent in *S. epidermidis*, aside from the 3' terminal transposon residues. The new *S. aureus* inserts apparently arose by transposition from *att554*; the corresponding *S. epidermidis* inserts clearly arose by transposition from a different parental site. Unearthing this parental site may provide clues as to the evolutionary history of Tn*554* in CNS.

The tight association of *att155* and Mc resistance in the outbreak *S. aureus* strains has been studied by Southern hybridization. Results indicate that the "extra" DNA that characterizes Mc^r *S. aureus*, and that harbors the Mc resistance determinant *mec*, also harbors *att155*.

CH 009 210 MODIFIED NUCLEOTIDES IN HUMAN RIBOSOMES: WHERE, HOW, WHY, AND SOME CONTRASTS WITH DNA METHYLATION, B. Edward H. Maden, Department of Biochemistry, University of Liverpool, Liverpool L69 3BX, U.K. Ribosomal RNA from man and several other vertebrate species contains some 210 modified nucleotides. This is almost three times as many modified nucleotides as the total number of nucleotides in a tRNA molecule. Most of the modifications comprise 2'-O-methyl groups (about 105 per ribosome), base methylations (about 10-12 per ribosome) and pseudouridines (about 95 per ribosome). The great majority of modifications occur very rapidly upon ribosomal precursor RNA but a few base methylations occur later during ribosome maturation. The early 2'-O-methylations play an essential but so far undefined role in ribosome maturation. A major current objective is to locate all of the many modified nucleotides in the rRNA primary and secondary structure. This task is now 60% complete. All of the methyl groups and most of the pseudouridines have been located in the 18S primary structure, and most of the methyl groups in the 28S rRNA primary structure. Almost all of the modified nucleotides that have been located are in the conserved structural core of rRNA, that is, in the regions whose general features of secondary structure are conserved between prokaryotes and eukaryotes. This is at first sight surprising since a high level of rRNA modification is a eukaryotic-specific phenomenon. The methyl groups are distributed between a wide variety of local sequences and secondary structures. In 28S rRNA there is a tendency for 2'-O-methyl groups to be partly clustered near domain boundaries, which are particularly complex regions of the structural core. *rRNA methylation differs from DNA methylation in several respects.* DNA methylation in vertebrates is base-specific and sequence-specific, being largely confined to the cytosine of CpG doublets, which are diad-symmetrical in DNA. Moreover, levels of DNA methylation are tissue specific and are related to levels of gene expression, whereas rRNA methylation appears to be largely tissue-invariant and related to the functional architecture of the ribosome. The molecular recognition events that define methylation sites and other modification sites in rRNA have yet to be understood in detail and possible approaches to this major challenge will be outlined.

Ribonucleoprotein Structure and Function

CH 010 **METHYLATED NUCLEOSIDES NEXT TO THE ANTICODON INFLUENCE THE EFFICIENCY OF THE tRNA OR ARE INVOLVED IN THE READING FRAME MAINTENANCE**, Glenn. R Björk, Anders S. Byström, Johanna U. Ericsson, Tord G. Hagervall and P. Michael Wikström, Department of Microbiology, University of Umeå, S-901 87 Umeå, Sweden

Effects on translation *in vivo* by modification deficiencies for 2-methylthio-N^6-(4-hydroxyisopententyl)adenosine (ms^2io^6A) or 1-methylguanosine (m^1G) in tRNA were studied in mutant strains of *Salmonella typhimurium*. Both methylated nucleosides are present on the 3'-side of the anticodon (position 37) in tRNA reading codons starting with U (ms^2io^6A) or C (m^1G). The ms^2io^6A has a profound effect on the efficiency of the anticodon-codon interaction as well as codon context sensitivity. The m^1G prevents the tRNA to shift the reading frame. It is postulated that the presence of the methyl group in guanine in position 37 prevents base pairing between position 37 and a C in the message. Since m^1G is present in all organisms at position 37 in a subset of tRNAs, our findings with a eubacterium apply to both eucaryotes and archaebacteria.

Functional Aspects of rRNA Methylation

CH 011 METHYLATION OF RIBOSOMAL RNA AS A CONTROL FOR PROTEIN SYNTHESIS, Gary A. Clawson, Julie Sesno, Kathy Milam, Yan Fei Wang, Department of Pathology, George Washington University, Washington, DC 20037

Many hepatotoxins inhibit protein synthesis as an early event in the course of injury. One of the earliest events following CCl_4 injury (which is centrilobular is a degranulation of ER, which corresponds with the early defect in protein synthesis. This CCl_4-induced defect is mediated through cytoplasmic hypomethylation of rRNA, which is specific for 2'O-ribose moieties, and remethylation of purified ribosomal subunits by S100 fractions restores their functional capacity. We have extended our investigations to galactosamine (GAL), a model for diffuse liver injury. This agent induces an early unexplained defect in protein synthesis which is seemingly independent of its later effects on RNA synthesis. GAL is also associated with a profound cytoplasmic hypomethylation of rRNA, which appears to be mediated through reductions in S-adenosylmethionine levels. The GAL-induced hypomethylatin therefore involves both 2'O-ribose and base moieties. Uridine, employed as a cytoprotective agent which significantly protects protein synthesis and rRNA methylation, appears to be associated with increased relative methylation of 2'O-H and 2'O-C and-profoundly decreased levels of 2'-G. Remethylation of hypomethylated ribosomes by cytoplasmic S100 fractions again seems to restore their functional capacity. We suggest that cytoplasmic methylation of ribosomal subunits is an important mechanism for control of protein synthesis in quiescent hepatocytes, and that hypomethylation is a common mechanism by which hepatocytes temporarily shut-down protein synthesis in response to injury.

CH 012 DIMETHYLATION OF ADENOSINES IN RIBOSOMAL RNA. FUNCTION AND GENETICS, Peter H. van Knippenberg, Bob van Gemen and Jeroen M. Bodlaender, Department of Biochemistry, Leiden University, Wassenaarseweg 64, 2333AL Leiden, The Netherlands.

Adenosine dimethylation (m^6_2A) in nucleic acids is, as far as we know, confined to two cases, both connected with antibiotic sensitivity or resistance. Two neighboring adenosines in the loop of a hairpin near the 3' end of 16S(-like) ribosomal RNA are dimethylated at the N6 position in almost all ribosomes. However, bacterial mutants resistant to kasugamycin lack these methyl groups due to a mutation in the ksgA gene that encodes the specific methylase. In a number of gram-positive bacteria a form of inducible resistance to erythromycin and other MLS type antibiotics is associated with dimethylation of the adenosine 2058 (E.coli numbering) in 23S rRNA. The 23S rRNA methylases are specified by erm genes. For several years we have studied the function and the genetics of the ksgA system in E.coli (compare ref.1 for a review). The $m^6_2Am^6_2A$ structure, located at the interface of the subunits, influences a number of steps in initiation and elongation of protein synthesis, including translational fidelity. This makes the assignment of a specific role for these methylgroups problematic. More recently we have studied the ksgA gene and the way it is expressed. The gene is transcribed from a weak promoter and surprisingly lacks a Shine-Dalgarno signal for translation. Regulation takes place at the level of translation by a mechanism that is similar to well established forms of autogenous repression of ribosomal protein genes. The methylases specified by the ksgA and erm genes are homologous. A detailed analysis by computer reveals that the erm methylases all contain internal deletions with respect to the ksgA methylase, suggesting that the latter enzyme is ancestral and that the erm genes arose by redirecting an already existing 16S rRNA modifying system in the direction of the 23S rRNA. Evidence will be presented to further support the relatedness of the two systems.

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DNA Methylation in Differentiation and Tumorigenesis

CH 013 STRUCTURE, FUNCTION, AND EVOLUTION OF MAMMALIAN DNA METHYLTRANSFERASE, Timothy H. Bestor, Department of Anatomy and Cellular Biology and Laboratory for Human Reproduction and Reproductive Biology, Harvard Medical School, Boston, MA 02115. Tissue- and sex-specific patterns of DNA methylation are involved in gene regulation and in the differential activity of the paternal and maternal genomes in development. Methylation patterns are established and maintained by DNA (cytosine-5)-methyltransferase (DNA MeTase). This enzyme displays only limited sequence specificity *in vitro*, and cell types with different methylation patterns contain indistinguishable forms of DNA MeTase; the nature of the factors which regulate DNA methylation *in vivo* is obscure at present. DNA MeTase has been purified from mouse leukemia cells, and the cDNA which encodes it has been cloned and sequenced. An open reading frame within the nucleotide sequence encodes a polypeptide of 1573 amino acids, a size consistent with the apparent mass of the largest species of DNA MeTase found in mouse cells. The protein consists of at least 2 distinct domains: a carboxyl-terminal domain of 570 amino acids that shows significant homology to bacterial type II DNA cytosine methyltransferases, and a 1000 amino acid amino-terminal domain. The amino-terminal domain contains a hydrophilic leader sequence rich in charged residues and a Cysteine-rich region that is similar to metal binding motifs found in other regulatory proteins. Antibodies raised against a synthetic peptide modeled after a sequence adjacent to the potential metal-binding region inhibits the activity of DNA MeTase. The amino- and carboxyl-terminal domains are joined by a run of alternating Glycine and Lysine residues that probably forms a flexible link or hinge between the domains. It appears that mammalian DNA MeTase may comprise a catalytic carboxyl-terminal methyltransferase domain joined to a regulatory amino-terminal domain that might mediate interactions between cellular factors and the catalytic domain to control the specificity of the enzyme *in vivo*. The implications of these finding for the evolution and function of mammalian DNA methyltransferase will be discussed.

CH 014 CELL MEMORY, X CHROMOSOME INACTIVATION, AND DNA METHYLATION, Arthur D. Riggs, Gerd Pfeifer, and Judith Singer-Sam, Department of Molecular Biology, Beckman Research Institute of the City of Hope, Duarte CA 91010

The molecular mechanisms for cell memory and X chromosome inactivation (XCI) will be discussed, and it is in this context that our experimental work will be presented. Both cell memory and XCI probably require higher order chromosomal structure which can be affected by DNA methylation. We have been studying the promoter and CpG-rich island of X-linked PGK. In vivo studies have shown that hypomethylation of this region correlates with the active state. At least three proteins bind to this region in vitro (Yang et al, Somat. Cell and Mol. Genet., 14:461-472, 1988). More recent studies have shown that deletion of a footprint region centered 130 base pairs upstream of the major transcription start site for PGK reduces five-fold transient expression in HeLa cells. Methylation of the promoter region reduces in vivo function four-fold, but, to date, in vitro protein-binding experiments have not shown an effect of methylation. In vivo footprinting and genomic sequencing studies on the PGK promoter and CpG island are currently underway. New assays for DNA methylation at specific sites and also for RNA levels have been developed and are being used to study changes during development. These assays, which use PCR methodology, are extremely sensitive and adequately quantitative; reproducibility of PCR signal strength is within about a factor of two over a thousand-fold range.

DNA Methylation and Gene Expression

CH 015 ON THE MECHANISM OF PROMOTER INACTIVATION BY SEQUENCE-SPECIFIC METHYLATION, Walter Doerfler, Miklos Toth, Ralf Hermann, Bernd Weisshaar, Dagmar Knebel-Mörsdorf, Arnd Hoeveler, and Pawel Dobrzanski, Institut für Genetik, Universität zu Köln, D-5000 Köln, Germany.

We have used the adenovirus E1A and late E2A promoters to demonstrate that sequence-specific methylations lead to the inhibition or inactivation of these promoters. For the late E2A promoter, inactivation by methylation at three 5'-CCGG-3' sequences has been shown in transient expression systems (1, 2), after fixation in the host genome (3) or in an in vitro transcription system (4) (5, for recent review). Promoter inhibition by DNA methylation can be, at least partly, overcome by the E1A 289aa transactivating protein of adenoviruses (2, 6) or by a strong enhancer near the methylated promoter (7).

By using the genomic sequencing technique (8), we have recently determined the patterns of methylation in all CpG residues of an active or an inactive late E2A promoter in transformed hamster cells. Methylation of the two downstream 5'-CCGG-3' sequences at positions +6 and +24 of the late E2A promoter compromises the binding of specific proteins. This interference might account in some ways for the inhibitory effect of promoter methylation.

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(Supported by the Deutsche Forschungsgemeinschaft through SFB74-C1)

CH 016 HORMONE DEPENDENT DNA DEMETHYLATION AND PROTEIN-DNA INTERACTION IN THE PROMOTER AND ESTRADIOL RESPONSE ELEMENT OF AVIAN VITELLOGENIN II GENE, Jost J.P., Saluz H.P., McEwan I., Feavers I.M., Hughes M., Reiber S. Friedrich Miescher Institut, PO Box 2543, CH 4002, Basel, Switzerland

Genomic sequencing and in vivo genomic footprinting have been used to study the state of methylation and the in vivo protein-DNA interaction on the promoter and the estradiol response element of avian vitellogenin II gene. In the promoter region, the demethylation of one CpG is strictly tissue and expression specific. The kinetics of demethylation on both DNA strands are parallel to the onset of vitellogenin mRNA synthesis. On the estrogen response element, the demethylation of two CpGs is estrogen dependent but not tissue specific. The kinetics of demethylation proceed in a strand-specific manner. The upper DNA strand becomes demethylated first followed 20 hours later by the demethylation of the lowest DNA strand. Correlated with the demethylation of the CpG in the promoter region is the appearance of a DNAse I hypersensitivity site and changes in the in vivo specific protein-DNA interactions. The in vivo data were corroborated by in vitro DNAse I footprinting and gel shift assays carried out with synthetic oligonucleotides and fractionated nuclear extracts from hens and rooster liver nuclei.In vivo genomic footprinting and in vitro DMS interference experiments on the ERE revealed only minor changes in the protection of the DNA before and after the stimulation of the gene by estrogen. Using specific monoclonal antibodies against estrogen receptor we demonstrate that the ERE binds also a protein different from estrogen receptor. The protein has been purified to homogeneity and characterized. It has a MW of 75 & 85 KD with a pI of 5. It is binding specifically to the ERE with a binding constant (Kd) of 5.10^{-11}M.

CH 017 METHYLATION PATTERN OF THE 5' FLANKING REGION OF THE MOUSE β GLOBIN (MAJOR) GENE, Cheryl Ward, Arthur Bolden and Arthur Weissbach, Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ 07110.

In vitro methylation of a cloned fragment of the 5' promoter region [-26 to -388] of the mouse β globin gene containing 3 CpG sites was carried out with a partially purified murine DNA methyltransferase. An asymmetric pattern of methylation was observed. The isolated CpG grouping at position (-300) is not methylated at all. Two CpG sites at positions -163 and -150, located just upstream of a known transcriptional regulatory site, CACACC, are methylated but at different rates and in different patterns. A partial sequence is shown below:

5'-CATCA CCG AAGCCTGATT CCGT AGAG CCACACCCTGG--
-163 -150

The upstream CpG site (-163) is methylated slowly on both strands in a symmetrical fashion. The CpG at -150 is methylated rapidly on only one strand to yield a hemimethylated site.

The *in vivo* methylation pattern of this regulatory region of the mouse β globin gene is also being examined in cultured mouse cells by genomic sequencing. Preliminary evidence suggests the presence of hemimethylated site in mouse erythroleukemia cells prior to the induction of β globin synthesis.

CH 018 TRANSCRIPTION OF A METHYLATED DNA VIRUS, Dawn B. Willis[1], James P. Thompson[2], Karim Essani, Rakesh Goorha, and Allan Granoff, Department of Virology and Molecular Biology, St. Jude Children's Research Hospital, Memphis, TN 38101

Frog virus 3 (FV3) and other closely related members of the Iridovirus family are unique among animal viruses in possessing a highly methylated DNA genome. All cytosines in the dinucleotide sequence dCpdG are methylated by a virus-encoded DNA methyltransferase whose activity depends on the presence of two polypeptide chains of 26 kD and 18 kD. Because FV3 uses host RNA polymerase II for early mRNA synthesis, it has evolved a mechanism for overriding the normal inhibition of DNA methylation on transcription. A plasmid containing the adenovirus 12 E1A promoter attached to the coding region for chloramphenicol acetyl transferase (CAT), shown by Kruzcek and Doerfler[3] to be inhibited by methylation with bacterial methylases, could be re-activated after transfection into HeLa cells that were subsequently infected with FV3, but only when viral protein synthesis was allowed to occur. Thus, the trans-acting protein that overcame the inhibitory effect of C-methylation on transcription was virus-induced, and not the same as the virion trans-acting protein required to turn on early FV3 mRNA synthesis. The promoter of one well-studied FV3 immediate early gene in a plasmid-CAT construct was mutated to alter 3 potentially methylatable sites to sites that could be methylated by bacterial methylases in vitro. This promoter - methylated or not - was activated by the FV3 virion protein, showing that methylation in this immediate-early gene was not at sites critical for transcription.

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[3] Proc. Natl. Acad. Sci. USA 80:7586 (1983)

Genomic Imprinting

CH 019 STUDIES ON THE METHYLATION PATTERN OF A PARENTALLY IMPRINTED MURINE TRANSGENE. J. Richard Chaillet,* Thomas F. Vogt,* Clarke F. Millette,# Judith L. Swain,@ and Philip Leder*. *Department of Genetics, Harvard Medical School, Boston, MA 02115, #Department of Anatomy and Cellular Biology, Harvard Medical School, Boston, MA 02115, @Department of Medicine, Duke University School of Medicine, Durham, NC 27710.

In a previously described transgenic mouse strain the pattern of transgene methylation correlates with parental inheritance and with expression of the transgene c-myc gene (1). Maternal inheritance produces a methylated and unexpressed transgene, whereas paternal inheritance results in reduced methylation and transgene expression in cardiac tissue. Further studies of this transgenic strain demonstrate that the paternally inherited pattern is not fully realized in male germ cells. There is a distinct transgene methylation pattern that is identical at all examined spermatogenic stages, regardless of the parental inheritance pattern of the surrounding somatic tissue. These results suggest two alternative models for the role of methylation in genomic imprinting.

The variability of parental influence on the methylation pattern of any particular molecular construct when placed into many transgenic strains (2,3) suggests that the locus of transgene integration influences the methylation pattern. Therefore, we examined the locus of integration on distal chromosome 16 of the previously described transgene (1). No evidence of a parental influence on the methylation pattern of this locus, in both adult and embryonic tissue, was found. The most likely explanation for this finding is that the structure of the integrated transgene is responsible, at least in large measure, for its parentally determined transgene methylation pattern.

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2. W. Reik, A. Collick, M. L. Norris, S. C. Barton, and M. A. Surani. Nature 328:248 (1987).
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Late Addition

CH 020 ***IN VITRO*** STUDIES ON THE METHYLATION OF *E. COLI* RIBOSOMES, James Ofengand, Didier Nègre and Carl Weitzmann, Roche Institute of Molecular Biology, Roche Research Center, Nutley NJ 07110-1199.

E. coli 16S RNA contains 10 methylated bases: m^7G524, m^2G966, m^5C967, m^2G1207, $m^4Cm1402$, m^5C1407, m^3U1498, m^2G1516, $m^6{}_2A1518$, and $m^6{}_2A1519$. There are no known phenotypes, mutants, or specific methylases for any of these residues except for A1518 and A1519 which are methylated by the same enzyme and for which mutants exist. None of the methylated bases are essential for any of the partial reactions of protein synthesis since functionally active unmethylated 30S ribosomes can be made from full-length 16S RNA made *in vitro* and a full complement of purified 30S ribosomal proteins (1). We now show that unmethylated 30S ribosomes and 16S RNA are excellent substrates for *in vitro* methylation with S-adenosyl methionine and cell-free extracts of *E. coli*. The methylases can be found both in association with ribosomes (1 M NH_4Cl wash) as well as free in the cytoplasm. Activities for the substrate-dependent synthesis of m^6A, $m^6{}_2A$, m^3U, m^2G, and m^5C were identified and partially purified. Strong preferences for either 30S ribosomes or 16S RNA were found. For example, m^2G formation was strongly biased toward 30S ribosomes, while the m^5C methylase strongly preferred 16S RNA. Although two m^5C residues are found *in vivo*, *in vitro* only one m^5C per RNA was obtained. This m^5C was identified as m^5C967. The specific methylation of m^5C967 at the RNA level suggests an early role in ribosome assembly. Work in progress includes (a) isolation, purification, and cloning of the m^5C967 methylase, (b) assessment of the effect of m^5C967 formation on 30S assembly and on function, and (c) extension of these studies to the other methylases and to *in vitro* synthesized 23S RNA.

1. Denman, R., Nègre, D., Cunningham, P.R., Nurse, K., Colgan, J., Weitzmann, C. and Ofengand, J. (1989) *Biochemistry* 28, 1012-1019.

mRNA

CH 100 A NUCLEAR (GUANINE-7-)METHYLTRANSFERASE ISOLATED FROM EHRLICH ASCITES CELLS, Guojun Bu and Thomas O. Sitz, Department of Biochemistry, Virginia Tech, Blacksburg, VA 24061.
An RNA(guanine-7-)methyltransferase has been partially purified from the nuclei of Ehrlich ascites cells. This enzyme at pH 8 bound to DEAE-Sepharose but did not bind to CM-Sepharose; at pH 7 it did bind to CM-Sepharose (the bound enzyme was eluted from both columns with about 0.15 M KCl). A molecular weight of 240,000 was determined by gel filtration on Sephacryl S-300. This enzyme had an apparent Km for S-adenosylmethionine of 13 μM. The substrate for this methyltransferase was methyl-deficient RNA isolated from the post-polysomal supernatant from mouse liver isolated from ethionine treated mice (M.D. post poly RNA). When the methylated RNA product was isolated and hydrolyzed with NaOH a fragment with a -5 charge was isolated, and when hydrolyzed with T2 RNase a fragment with a charge of about -4.5 was isolated. The 7-methylguanine purine ring opens at high pH and the positive charge at the 7-position is lost. After digestion with venom phosphodiesterase and alkaline phosphatase we identified 7-methylguanosine as the methylated component in the "cap" structure. Post-polysomal RNA from mouse liver was not a substrate, while post-polysomal RNA isolated from Ehrlich ascites cells had about half the methyl-accepting activity of RNA isolated from ethionine treated mouse liver. Therefore, in hypomethylated tissue we find non-methylated cap structures in RNA that serves as substrate for this methyltransferase. Commercial "cap" structures such as GpppN were inefficient methyl-acceptors for the (guanine-7-)methyltransferase.

CH 101 N^6-METHYLADENOSINE OCCURS IN AN INTRON-SPECIFIC REGION OF PROLACTIN RNA AND UNDERMETHYLATION INFLUENCES THE NUCLEAR PROCESSING OF THIS PRECURSOR, Simon M. Carroll, Prema Narayan and Fritz M. Rottman, Department of Molecular Biology and Microbiology, Case Western Reserve University, Cleveland, OH 44106.
An in vitro methylation reaction was used to predict the presence of methylated adenosine (m^6A) residues at specific sites within an intron region of a bovine prolactin (PRL) precursor RNA. Capped synthetic transcripts of an mRNA precursor molecule were incubated in a nuclear extract in the presence of the methyl donor, [^{3}H] S-adenosylmethionine. The intron-specific region was hybrid-selected and high pressure liquid chromatography analysis indicated that all the [^{3}H]-labeled material in the intron was present as m^6A. Furthermore, when T1 oligonucleotides were separated by polyacrylamide gel electrophoresis only oligonucleotides containing the consensus sequences GAC and AAC were identified as possessing m^6A. To investigate if the intron was also methylated in vivo the intron-specific region was hybrid-selected from Chinese Hamster Ovary (CHO) cells containing a modified PRL gene. Examination of the T1 oligonucleotides of the intron specific region indicated that only oligonucleotides with GAC and AAC sequences possessed m^6A residues.

Using the methylation inhibitor, Neplanocin A(NPC), the effect of undermethylation on nuclear processing and cytoplasmic appearance of PRL mRNA has been examined. Monolayers of CHO cells stably transfected with a PRL minigene containing the last intron were treated with 10uM NPC. Undermethylation of m^6A residues in the poly A^+ RNA pool mRNA was correlated with the delayed cytoplasmic appearance of PRL mRNA. Furthermore, when steady-state levels of the nuclear precursor were measured, NPC treated cells contained four to six times more precursor molecules relative to mature form RNA than did control cells. Therefore, undermethylation of the PRL precursor is accompanied by a significant delay in the cytoplasmic appearance of mature form RNA and an accumulation of the precursor molecule.

CH 102 ANTIVIRAL ACTIVITIES OF 3-DEAZAADENOSINE ANALOGS--INDIRECT INHIBITORS OF METHYLATION, Peter K. Chiang, Department of Applied Biochemistry, Walter Reed Army Institute of Research, Washington, DC 20307-5100.
3-Deazaadenosine and 3-deaza-(±)aristeromycin are inhibitors of S-adenosylhomocysteine hydrolase. When cells are treated with these two inhibitors, cellular levels of S-adenosylhomocysteine increase due to the inhibition of S-adenosylhomocysteine hydrolase. The accumulation of S-adenosylhomocysteine in turn leads to the inhibition of S-adenosylmethionine-dependent methylation reactions. HeLa cells infected with vaccinia virus were treated with 3-deaza-(±)aristeromycin, 3-deazaadenosine, or sinefungin. 3-Deaza-(±)aristeromycin was the most effective in inhibiting the plaque formation of vaccinia virus; 99.8% inhibition at 4 uM without showing any cytotoxicity. In comparison, 3-deazaadenosine and sinefungin showed much less favorable therapeutic indices. The former inhibited vaccinia virus placque formation by 93.4% at 30 uM while the HeLa cells were only 78% viable; the latter inhibited plaque formation by 89.6% at 200 uM while the cells were 90% viable. Both 3-deaza-(±)aristeromycin and 3-deazaadenosine had no effect on [^{3}H]thymidine incorporation into total HeLa DNA or viral (cytoplasmic) DNA. Upon examination by sucrose density gradient centrifugation, however, the newly synthesized viral DNA was found not to be packaged into intact virus. No evidence was found that the methylation of the 5' cap of vaccinia mRNA was inhibited by 3-deaza-(±)aristeromycin. 3-Deaza-(±)aristeromycin could, presumably, exert its antiviral effect by inhibiting other vital methylation reaction(s) essential for the packaging of functional vaccinia virus.

CH 103 EVIDENCE THAT THE SINDBIS VIRUS RNA METHYLTRANSFERASE IS ASSOCIATED WITH THE VIRAL NONSTRUCTURAL PROTEIN, NSP1. Sha Mi[1], Russell Durbin[2], Vincent Jung[1], Victor Stollar[1]. [1]UMDNJ-Robert Wood Johnson Med. Sch., Piscataway, NJ 08854, and [2]SUNY-Health Sciences Center, Brooklyn, New York 11203.
Standard Sindbis virus (SV) is unable to grow in *Ae. albopictus* cells deprived of methionine. The mutant SV_{LM21} is able to grow under these conditions (i.e., has a low methionine-resistant [LMR] phenotype) because it generates an RNA methyltransferase with an increased affinity for S-adenosylmethionine (ado met). By substituting the nucleotide sequence 125-504 from SV_{LM21} cDNA for the corresponding sequence of the Toto 1101 plasmid (infectious Sindbis viral RNA can be transcribed from this plasmid) we were able to generate recombinant virus with the LMR phenotype. Sequencing of the nucleotide 125-504 region of SV_{LM21} RNA revealed two mutations; these occurred in adjacent codons and lead to two predicted amino acid changes in the SV nsP1 protein; at residue 87 from Arg to Leu, and at residue 88 from Ser to Cys. Since the nucleotide sequence 125-504 occurs entirely within the gene for nsP1, we propose that the RNA methyltransferase activity generated by SV is associated with the nonstructural protein, nsP1. Site-directed mutagenesis was used to produce virus with only the change at residue #87 or only the change at residue #88; in both cases the virus exhibited partial LMR phenotype. We are currently working on the expression of the Sindbis virus NSP-1 proteins.

CH 104 STRUCTURE AND FUNCTION OF YEAST mRNA CAPPING ENZYME
K. Mizumoto, Y. Shibagaki, N. Itoh, H. Yamada, S.Nagata, and Y. Kaziro
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Capping enzyme from rat liver and Artemia salina consists of a single polypeptide chain with an approx. Mr of 70,000 containing two catalytic domains for each of the initial two consecutive reactions involved in the cap formation, i.e., those catalyzed by mRNA guanylyltransferase and RNA 5'-triphosphatase. In contrast, the highly purified yeast capping enzyme is composed of two separate chains of 52kDa (α) and 80kDa (β), responsible for the activities of guanylyltransferase and triphosphatase, respectively. To see whether the α and β subunits are derived from a single precursor polypeptide or are synthesized as independent polypeptides encoded by two separate genes, we attempted to isolate the gene(s) for the yeast capping enzyme. An yeast genomic expression library in λgt11 was screened with an antibody against yeast capping enzyme. One of the positive clones, λC3, contained a 3,500 bp yeast DNA insert. From experiments based on the affinity selection of the antibodies by antigens produced with λC3 in E. coli, this clone was found to contain the gene for α subunit. The insert contained an open reading frame encoding a polypeptide chain with a calculated Mr of 52,746 which fairly corresponds to the size of the α chain. The identity of the gene was further confirmed by expressing the gene in E. coli to give a catalytically active guanylyltransferase. From the primary structure of this gene and from its mRNA size, we concluded that the α and β chains of yeast capping enzyme are encoded by two separate genes. Disruption of one copy of the α subunit gene in a diploid yeast created a recessive lethal mutation, indicating that the single guanylyltransferase gene of yeast has an essential function.

CH 105 CHARACTERIZATION OF SUBSTRATE REQUIREMENTS FOR N^6-METHYLADENOSINE FORMATION IN PROLACTIN mRNA, Prema Narayan and Fritz M. Rottman, Department of Molecular Biology and Microbiology, Case Western Reserve University, Cleveland, OH 44106.
The N^6-methyladenosine residues in bovine prolactin mRNA are found predominantly in the 3' region of the molecule. This region of 138 nucleotides contains three AAC or GAC consensus methylation sequences out of a total of 27 such sequences present in the entire mRNA. A cell free system has been developed which is capable of methylating an in vitro generated prolactin transcript at internal adenosine residues. Of the three consensus sequences, only the adenosine residue present in the GAC sequence is methylated in vitro. Furthermore, this is the same adenosine residue that is methylated in vivo in prolactin mRNA isolated from bovine pituitary. This high degree of specificity of methylation indicates that the methyltransferase likely recognizes more than just the 3 consensus nucleotides. The sequence around the methylated adenosine is PuGACU, indicating that this may be the preferred methylation sequence. To further characterize the primary sequence requirements for the methyltransferase, we have utilized the 138 nucleotide segment of this mRNA as a substrate for the enzyme. This sequence is accurately methylated at the same adenosine as the full length mRNA substrate. Utilization of these small RNA molecules as substrates for methylation allows a more precise definition of the contribution of additional nucleotides to the core consensus sequence and its recognition by the methyltransferase.

CH 106 *IN VITRO* METHYLATION OF *E. COLI* 16S RNA AND 30S RIBOSOMES. Didier Nègre, Carl Weitzmann and James Ofengand, Roche Institute of Molecular Biology, Roche Research Center, Nutley NJ 07110 USA.
Full-length 16S rRNA lacking all of the normally methylated nucleotides can be synthesized *in vitro* and assembled with ribosomal proteins to form active 30S subunits (Krzyzosiak *et al.*, 1987, *Biochemistry* **26**, 2353-2364). Treatment of the synthetic 16S RNA or 30S particles with S-adenosyl-[^{3}H]methionine and either an S100 or ribosomal high salt wash (HSW) extract resulted in RNA or ribosome-dependent incorporation of [^{3}H]methyl groups into TCA-insoluble material. Enzymatic hydrolysis of labeled RNA to nucleosides followed by HPLC analysis identified the [^{3}H]methylated bases. Activities for the formation of m^6A, m_2^6A, m^5C, m^7G and m^2G residues were detected in both extracts. Fractionation by ammonium sulfate partially resolved the different activities. All of the fractions with m^5C activity were 6-8 times more active on 16S RNA than on 30S ribosomes, while the m^2G activity preferred 30S ribosomes by a factor of more than 10. The m_2^6A (and m^6A) activity was ribosome-specific, but the m^7G activity did not distinguish between the two substrates. Whereas two m^5C residues are found *in vivo* at residues 967 and 1407, the m^5C activity present in the 55-85% ammonium sulfate fraction of HSW yielded only a maximum of 1.0 mole m^5C/mole 16S RNA. RNase protection by oligodeoxynucleotide hybridization identified the residue as exclusively C967. The availability of unmethylated synthetic 16S rRNA and 30S ribosomes and the demonstration that they can function as effective methylase substrates makes possible (a) isolation and characterization of the methylases, (b) determination of when during ribosome assembly a given methylation occurs, and (c) what effect that methylation has on ribosome assembly and function.

CH 107 THE ROLE OF RNA METHYLATION IN THE DIFFERENTIATION OF THE L5 MYOBLAST LINE, Sigfrido Scarpa, Laura Di Renzo and Roberto Strom, Dipartimento di Biopatologia Umana and 1° Istituto di Clinica Chirurgica, Università di Roma "La Sapienza", Rome, Italy.
The L5 myoblast cell line can be maintained, in a "growth medium" (with 10% FCS), in the undifferentiated state for many passages, or be induced, by transferring the cells in a "fusion medium" (with 1% FCS), to differentiate into multinucleated fibers. We decided to investigate the possible changes in RNA methylation levels under fusing and non-fusing conditions, and by adding 3-deazaadenosine (DZA), a potent inhibitor of methylation processes which stimulates L5 myoblast differentiation, or Concanavalin A (Con-A), which has been reported to increase RNA methylation in T-lymphocytes and which inhibits L5 myoblast differentiation. Cells were treated with DZA or Con-A the day after plating and maintained in the presence of the drugs throughout the duration of the culture. The cells, under conditions of inhibition of both de novo purine synthesis and methylation via the tetrahydrofolate pathway, were labeled with (^{3}H-methyl)-methionine and ^{14}C-uridine, and total RNA was purified. In growth medium, an increase or a decrease in the extent of total RNA methylation did not induce per se L5 myoblast differentiation. On the other hand, in fusion medium the RNA methylation levels appeared to be inversely related to the induction of the differentiation process. RNA rate of synthesis decreased as well, but not as markedly, with culture ageing.

CH 108 THE EFFECT OF HYPERMETHYLATED AND NON-STANDARD 5'-CAP STRUCTURES ON mRNA ACTIVITY, Stanley M. Tahara, Edward Darzynkiewicz, Janusz Stepinski, Irena Ekiel, Chang Hahn, James H. Strauss, and Teju Sijuwade, Dept. of Microbiol., USC School of Medicine, Los Angeles, CA 90033-1054, Dept. of Biophysics, Inst. of Exptl. Physics, U. of. Warsaw, Poland, Dept. of Chem., U. of Warsaw, Poland, Biotechnology Res. Inst., Nat. Res. Council of Canada, Montreal, Canada, and Div. of Biology, Caltech, Pasadena, CA 91125
Capped rabbit β-globin mRNAs containing aberrant 5'-cap structures were synthesized using T7 RNA polymerase and β-globin cDNA template in the presence of different extended caps, *i.e.* dinucleotides of the form NpppG [N=7-methyl guanine (m^7G), 7-ethyl G (e^7G), 7-benzyl G (bn^7G), 2,7-dimethyl G ($m_2^{2,7}G$), or 2,2,7-trimethyl G ($m_3^{2,2,7}G$)]. All extended caps were correctly incorporated into transcripts based on 5'-end analyses. When incubated in reticulocyte lysate, these mRNAs exhibited different efficiencies. Messengers capped with bn^7G or $m_2^{2,7}G$ were observed to be up to 2X more active than m^7G capped mRNAs. RNAs capped with e^7G were less active than m^7G-capped counterparts, whereas $m_3^{2,2,7}G$ capped mRNA was inactive. The effect of $m_2^{2,7}G$ and $m_3^{2,2,7}G$ cap structures on translation of Sindbis 26S mRNA was also examined. These hypermethylated cap structures are found on a significant percentage (up to 50%) of Sindbis and other togavirus 26S mRNAs during infection. The activity of 26S mRNA, *in vitro*, was found to be enhanced when capped with $m_2^{2,7}G$ as compared to the standard m^7G cap. By contrast, $m_3^{2,2,7}G$ capped 26S mRNA was virtually inactive. These results suggested a potential role of $m_2^{2,7}G$ caps as positive elements in viral mRNA translation. Hypermethylated 26S mRNAs may have different translation activities *in vivo*. The different activities of di- and trimethylated mRNA 5'-caps appear to be a general effect, not limited to a specific mRNA. [SMT was supported by Amer. Cancer Soc. (MV-257), LHI Med. Res. Fund, and NIH (GM38512); E. D. was supported by CPBR-3.13 from Polish Academy of Sciences and CPBR-01.06 from Ministry of Eucation of Poland].

CH 109 6-METHYLADENINE LEVELS IN DIHYDROFOLATE REDUCTASE MESSENGER RNA
Martin T. Tuck and Amy P. Rana, Department of Chemistry, Ohio University, Athens, OH 45701.

RNA transcripts coding for the enzyme Dihydrofolate Reductase (DHFR) were purified from the methotrexate resistant mouse sarcoma cell line S180-500R. This cell line contains approximately 500 fold more DHFR mRNA than it's unselected counterpart due to an amplification of the DHFR gene. The transcripts were purified from the poly A+ RNA fraction of the cells using selective hybridization techniques. The purified transcripts were then base hydrolyzed and the nucleosides 3' end labeled using γ-^{32}P-ATP and T4 polynucleotide kinase. The labeled nucleotide mixture was then analyzed for 6-methyladenine residues using high pressure liquid and two dimensional thin layer chromatography. In addition, the multiple forms of the DHFR transcripts (which differ mainly in the 3' end polyadenylation site) were separated using sucrose density centrifugation and each subfraction analyzed for 6-methyladenine content. Preliminary nuclease mapping experiments using protected DNA fragments complementary to different regions of the transcript showed the coding region to be highly enriched in 6-methyladenine.

DNA Methylation in Prokaryotes, Fungi and Plants

CH 200 SITE-DIRECTED MUTAGENESIS OF THE *Eco*RV METHYLASE FOR THE STUDY OF THE FUNCTIONAL ROLE OF THE CONSERVED SEQUENCES IN N^6-ADENINE METHYLASES, Tanya Barshevsky and Jack S. Benner, New England Biolabs, Beverly, MA 01915.

*Eco*RV methylase has been used as a model system to investigate the function of the conserved sequences in N^6-Adenine methylases. The *Eco*RV methylase gene was subcloned and the methylase protein was purified by multiple chromatographic steps. The highly conserved BPPY protein sequence in the *Eco*RV methylase was changed using site-directed oligonucleotide mutagenesis in both conservative and radical ways. We produced protein sequence changes at the D, the first P and the Y so that the predicted secondary and unknown tertiary structures of this region were not only gently perturbed but also disrupted. The *Eco*RV methylase with the first change (DPPY to EPPY) was subcloned back into a modified pUC19 vector . This change produced a methylase which unlike the wild-type enzyme does not fully methylate *in vivo* the genomic or vector DNA. We are examining the catalytic properties of the wild-type *Eco*RV methylase and some mutagenized methylase proteins. Also efforts will be made to determine whether SAM or DNA binding, if any, remains in mutant methylases.

CH 201 THE PURIFICATION AND CHARACTERIZATION OF BstNI MODIFICATION METHYLASE,
Ya.I. Buryanov, M.M. Baryshev, V.G. Kosykh, A.A. Bayev, Institute of Biochemistry and Physiology of Microorganisms, USSR Academy of Sciences, Pushchino, Moscow region, 142292, USSR

We have isolated and purified the BstNI modification methylase (M.BstNI) from Bacillus stearothermophilus. This enzyme catalyses the transfer of methyl groups from AdoMet to CCA/TGG DNA sequences yielding N4-methylcytosine. This modification renders the DNA resistant to cleavage by both BstNI and EcoRII restriction endonucleases. At the same time M.BstNI is able to modify the DNA previously methylated by EcoRII methylase while the DNA methylated by M.BstNI is resistant to EcoRII methylation. This property of two methylases can be used for analysis of the 5-methylcytosine presence in plant DNA in the CCA/TGG sequence. We have partially purified M.BstNI by DEAE-cellulose, phosphocellulose and hydroxylapatite column chromatography. The enzyme has a molecular weight of 60 kD as determined by gel-filtration and SDS-polyacrylamide gel electrophoresis. E. coli clones were isolated from libraries of B. stearothermophilus DNA fragments in pUC19 by selecting for self-modified molecules that were resistant to BstNI endonuclease digestion.

CH 202 MOLECULAR NATURE OF THE RESTRICTION-MODIFICATION SYSTEM LT OF *Salmonella typhimurium*, Olivier De Backer and Charles Colson, Unité de Génétique (GENE), Université de Louvain, B-1348 Louvain La Neuve, Belgium.
Restriction-Modification (R-M) system LT is present in most *Salmonella* strains. Its genes are chromosomally located near *proC*. We have cloned these genes and determined some properties of the coded enzymes. On appropriate *S. typhimurium* strains, the phage vector λ EMBL4 was very strongly restricted by LT. This allowed the selection of a λ clone carrying the modification gene and therefore immune to the LT restriction. This gene was subcloned into plasmid vectors and expressed in *E. coli*. A restricting recombinant clone was isolated from a plasmid genomic library of *S. typhimurium*. made in a modifying host strain. This clone proved to contain a plasmid harboring the genes coding for both restriction and modification activities. In contrast with other studied R-M systems, the introduction of LT genes into a non-modifying host is lethal, probably because of self-restriction of the chromosomal DNA. The sequence of the recognition site of the LT enzymes was foud to be 5' GAGAC 3'. It is characteristic of type III R-M systems (5 bp long, asymmetric, adenine present on only one strand). The methylated base is the 5' adenine.

CH 203 A METHYLATION-SPECIFIC DNA-BINDING PROTEIN FROM PLANTS DIFFERS FROM THAT OF MAMMALIAN TISSUES. K.C. Ehrlich, D. Zhang, P. Supakar, and M. Ehrlich, Southern Regional Research Center and Department of Biochemistry, Tulane School of Medicine, New Orleans, LA 70179. A family of proteins (DBP-m) isolated from nuclear extracts of various plant species binds selectively to DNA containing one or more 5-methylcytosine (m^5C) residues. The binding properties of this protein were compared to those of the only other known methylated DNA-binding protein, MDBP. MDBP has been isolated from a variety of mammalian cells. Binding to DBP-m was measured by the band-shift assay using synthetic oligonucleotides containing 17 to 39 base-pairs including one or more m^5C residues. The ability of DBP-m to bind to methylated ligands was compared to its ability to bind to the unmethylated analogs. Binding of DBP-m to m^5C-rich oligonucleotide duplexes is up to 20-fold greater than to the analogous unmethylated duplexes, whereas, for MDBP, the preference can be greater than 250-fold. The extent of binding increases when the oligonucleotide contains more than one m^5C residue but one suffices for binding. For DBP-m, the longer oligonucleotides duplexes bound much better than shorter ones when the number of m^5C residues remained the same. In contrast, for MDBP, binding was only slightly better with the longer duplexes. Both DNA-binding proteins showed no detectable binding to single-stranded oligonucleotides. Unlike MDBP, plant DBP-m binds to DNA without apparent sequence-specificity and T residues cannot be substituted for m^5C in the DNA recognition sites. Evidence is emerging for a role of MDBP in control of transcription. Whether DBP-m also has such a role despite its lack of sequence-specificity remains to be determined. Supported in part by USDA grant, 85-CRCR-1-1751.

CH 204 CHARACTERIZATION OF CLONES OF THE BamHI METHYLTRANSFERASE GENE, Peter D. Nathan, and Joan E. Brooks, New England Biolabs, 32 Tozer Road, Beverly, MA 01915.
The BamHI type II restriction-modification system from Bacillus amyloliquefaciensH, recognizes the sequence 5'-GGATCC. We are characterizing different subclones of the BamHI methyltransferase (MTase) gene. One clone carries a 2.2-kb HindIII fragment (pBamM2.2) that contains the MTase gene and the N-terminal end of the endonuclease gene. A second clone carries a 1.8-kb XmnI- HindIII fragment (pBamM1.8) that contains the MTase gene and a portion of the ORF located in the intergenic region. These two clones differ in their compatibility with McrB (modified cytosine restriction). Only the plasmid pBamM1.8 is restricted by McrB+ hosts. Two approaches are being used to investigate the different McrB compatibilities. First, we have have found that cells containing pBamM1.8 produce significantly greater amounts of BamHI MTase than cells containing pBamM2.2. Second, we have found that a disruption of plasmid pBamM2.2 in the intergenic ORF results in the loss of McrB compatibility. Together, these results suggest that the higher level of MTase from pBamM1.8 is responsible for the McrB restriction, and that the region located between the two genes affects the level of MTase produced.

CH 205 PREDICTIVE MOTIFS OF CYTOSINE METHYLASES, Janos Posfai and Richard J. Roberts, Cold Spring Harbor Laboratory, P.O.Box 100, Cold Spring Harbor, NY 11724

Fourteen bacterial DNA methyltransferases that catalyze the formation of 5-methylcytosine within specific DNA sequences possess related structures. Similar building blocks (motifs), containing invariant positions, can be found in the same order in all fourteen sequences. Five of these blocks are highly conserved while a further five contain weaker similarities. One block, which has the most invariant residues, contains the proline-cysteine dipeptide of the proposed catalytic site. A region in the second half of each sequence is unusually variable both in length and sequence composition. Those methyltransferases that exhibit significant homology in this region share common specificity in DNA recognition. The five highly conserved motifs can be used to discriminate the known 5-methylcytosine forming methyltransferases from all other methyltransferases of known sequence, and from all other identified proteins in the PIR, GenBank and EMBL databases. These five motifs occur in the eukaryotic mammalian methyltransferase. By searching the unidentified open reading frames present in the GenBank and EMBL databases two potential cytosine methyltransferases have been found that were not recognized previously.

CH 206 VARIATION IN METHYLATION LEVELS OF SATELLITE DNA SEQUENCES IN THE CELL CULTURES OF BRASSICA JUNCEA (L.) CZERN, A.S.REDDY AND SIPRA GUHA-MUKHERJEE, SCHOOL OF LIFE SCIENCES, JAWAHARLAL NEHRU UNIVERSITY, NEW DELHI-110 067, INDIA. Satellite DNA was isolated from hypocotyls(H), proliferating callus(PC) and differentiating callus(DC) of Brassica juncea. All the three satellite DNAs were GC rich and contain high 5-methylcytosine(m^5Cyt) content than cytosine. Higher percentage of methylation and m^5Cyt/Cyt ratio were observed in H satellite DNA when compared to PC and DC satellites. To find out methylation pattern, total nuclear DNAs of H,PC and DC were digested with HpaII and MspI and hybridized with a cloned 200bp HindIII repeat unit of H satellite DNA. H satellite DNA can be partially digested with MspI but not by HpaII. In contrast, the PC satellite DNA could be hydrolyzed by both the enzymes, where as DC satellite DNA was found to have become partially resistant to HpaII and MspI. The results indicate that the PC satellite DNA was undermethylated, where as DC and H satellite DNAs were partially and highly methylated respectively. The majority of CCGG sequences of the satellite DNAs were not digested by either of the enzymes due to double methylation(mCmCGG). The results suggest that demeth ylation of some of the satellite DNA sequences appears to be an essential step before other regulatory steps leading to proliferation and differentiation of explants can occur in in vitro conditions.

CH 207 STRUCTURE-FUNCTION ANALYSIS OF ENZYME-DNA INTERACTIONS IN THE ECO RI DNA METHYLASE, Norbert O. Reich, Neda Mashhoon, Elizabeth Everett, and Michael Danzitz, Chemistry Department, University of California, Santa Barbara, CA 93106

Our goal is to understand the molecular basis of DNA sequence recognition and the modulation of this process by the cofactor S-adenosylmethionine, [AdoMet] in this prokaryotic system.

The kinetic mechanism and the rate determining step[s] have been elucidated: AdoMet binds first, followed by the double stranded DNA substrate [5'GAATTC3']. Rapid transfer of the methyl group to the DNA in the ternary methylase-AdoMet-DNA complex is followed by rate limiting step[s].

One portion of the methylase involved in AdoMet binding has been identified: it is a flexible peptide connecting two stable domains. This flexible hinge region and the domains were characterized using photoaffinity analogs, *in vitro* proteolysis, and peptide sequencing.

Hydroxy radical footprinting and synthetic oligonucleotides have been used to characterize the methylase-DNA topology. Moreover, DNA substrates with modified bases [uracil, inosine, deaza-adenine, etc.] have been submitted to comparative specificity analysis [k_{cat}/K_M]. Data from both analyses will be presented; the contribution to overall specificity deriving from individual methylase-DNA interactions has been elucidated.

CH 208 DNA DEMETHYLATION BY 5-AZACYTIDINE INDUCES DWARLISM IN RICE, H. Sano, I. Kamada, S. Youssefian and H. Wabiko, Biotechnology Institute, Akita Prefectural College of Agriculture, Akita 010-04, Japan. A single exposure of rice seedlings to 5-azacytidine (azaC) or 5-azadeoxycytidine (azadC) induced dwarfism, but other nucleotide analogues, AZT, 6azaT, 8azaG and araC did not show any effects on plant growth. The seedlings were most sensitive to azaC during the first 6 days of germination. The duration of azaC treatment determined the proportion of dwarfs, but not the extent of dwarfism. Seedlings treated with azaC exibited normal characteristics at maturity as untreated controls, except that their height was reduced by about 15%. The F_1 progeny obtained by self-fertilization also showed the dwarf characteristics. Genomic DNA isolated from both seedlings and mature leaves of azaC-treated seedlings had about a 15% reduction in the 5-methylcytosine content. Southern analysis using an alcohol dehydrogenase cDNA and a 960bp rice repetitive sequence indicated a reduced level of DNA methylation in azaC treated plants. The in vitro translation products of mRNA isolated from azaC-treated plants differed from those of untreated controls, indicating that azaC affected gene expression. The demethylation patterns of genomic DNA were also identified in the F_1 progeny. Thus demethylation and dwarfism of azaC-treated plants were heritable. The results suggest that azaC induced demethylation of genomic DNA, that caused an altered pattern of gene expression. Such changes in gene expression most probably resulted in a reduction in plant height.

CH 209 MOLECULAR CLONING AND CHARACTERIZATION OF THE GENE ENCODING THE CYTOSINE METHYLTRANSFERASE, M.CviJI, FROM CHLORELLA VIRUS IL-3A

Shelly L. Shields, Dwight E. Burbank and James L. Van Etten, Department of Plant Pathology University of Nebraska, Lincoln, NE 68583-0722

The gene encoding the DNA methyltransferase, M.CviJI from Chlorella virus IL-3A was cloned in pUC19 and expressed in E. coli RR1. Recombinant plasmid pIL-3A.22.8 containing a 3.6 kbp KpnI/Sau3A restriction fragment encoding M.CviJI methylates the internal cytosine in PuGCPy sequences or a subset of PuGCPy sequences in vivo. Methylation of these sequences by M.CviJI prevents digestion of pIL-3A.22.8 by restriction endonucleases sensitive to cytosine methylation in PuGCPy recognition sequences. Transposon Tn5 mutagenesis localized the M.CviJI functional domain on pIL-3A.22.8. Restriction fragments from the HpaI restriction site, 185 bp from the termini of the terminal repeat of Tn5, to the SalI and KpnI restriction site of pIL-3A.22.8 polylinker were isolated from individual Tn5 mutants. These restiction fragments containing the functional domain and flanking sequences were subcloned in single stranded sequencing vectors M13mp18 and M13mp19 for nucleotide sequencing. The amino acid sequence deduced from M.CviJI nucleotide sequence was compared to the amino acid sequence of 5-methylcytosine methyltransferases from prokaryotes to determine potentially shared protein domains. The M.CviJI gene was not essential for IL-3A replication since a M.CviJI deletion mutant also replicated in Chlorella.

CH 210 DEMONSTRATION OF 5-METHYL CYTOSINE OCCURRENCE IN THE GENOME OF *ASPERGILLUS NIDULANS*. Tamame M and Santos E. Institute of Microbiology-Biochemistry, CSIC, Univ. of Salamanca, Spain and Laboratory of Molecular Microbiology, NIAID, NIH. Bethesda MD 20892

The ascomycete fungus A. nidulans has been reported to lack genomic methylation because global genomic analysis using different techniques consistently yielded undetectable levels of 5-methylcytosine (5-MC) or N^6-metyladenine (N^6-mA). We have previously shown in this fungus that 5-azacytidine (5-AC) specifically modifies the *fluF* gene thus leading to heritable developmental alterations (fluffy phenotype). The molecular alteration(s) caused by 5-AC at the *fluF* locus is unknown.

We obtained stable normal revertants from fluffy variants through integrative transformation of cosmid clones of wild type DNA at a specific site in their genome. In order to test for the possibility of methylation within specific cellular sequences, we performed Southern analysis of fluffy, revertant and wild type genomic DNA using fragments of the rescuing cosmid as probes in combination with pairs of isoschizomeric enzymes able to distinguish between methylated forms of their recognized sequences.

Our results demonstrate the occurrence of 5-mC at specific sites of the A. nidulans genome encompassing sequences of the types GATC*, CC*AGG and CC*GG. No evidence has been found for the occurrence of N^6-mA.

These results constitute the first direct report of genomic methylation in Aspergillus and underscore the need for using bacterial hosts not altering the methylated state of cloned DNA in studies of the *fluF* locus.

CH 211 MUTATIONS IN METHYL-DIRECTED MISMATCH REPAIR PERMIT INTERSPECIES RECOMBINATION BETWEEN ESCHERICHIA COLI AND SALMONELLA TYPHIMURIUM. David S. Thaler* Christane Raysseguier# and Miroslav Radman# *Biology Department University of Utah Salt Lake City UT 84112 #Institut Jacques Monod C.N.R.S. Universite Paris VII 2 Place Jussieu Tour 43 75251 Cedex 05 Paris France. Escherichia coli and Salmonella typhimurium are closely related and, inside protein encoding sequences, are about 80% homologous at the DNA level. Nevertheless, generalized recombination between these species is normally very rare. We have found that if a Salmonella recipient is null for any of several genes, previously identifed as involved in methyl-directed mismatch repair, then the recipient is able to recombine much more efficiently with introduced "homeologous" (i.e. homologous, but with a mismatch about every 10 or 20 nucleotides) E. coli sequences. The enhanced recombination is seen with mutL, mutS, and mutH; and is observed whether the E. coli DNA is introduced by conjugation or by phage transduction. These findings support models in which homeologous recombination involves a reversible heteroduplex intermediate that is detected and aborted through a process that involves recoginition of the frequent mismatches.

CH 212 DNA METHYLATION IN WHEAT SEEDLINGS. Boris F. Vanyushin and Michail D. Kirnos, A.N.Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry, Moscow State University, Moscow 119899, USSR
The periodic synchronous synthesis of nuclear DNA in initial leaf of wheat seedlings was detected. This corresponds to S phases in leaf meristem cells and was exploited for investigation of DNA methylation in the cell cycle. Replicative and postreplicative DNA methylations are well discriminated. They are different in site specificity, sensitivity to phytohormones and inhibitors of DNA methylation. Newly synthesized DNA are undermethylated and highly asymmetrical for 5-methylcytosine content in complementary strands. This asymmetry diminishes as a result of postreplicative methylation up to end of cell cycle. Hence, in dividing cells the early and lately replicated genes can be discriminated. In the presence of 5-azacytidine the second replication cycle is strongly inhibited and third one does not appear. Thus, methylation may control DNA replication in plants. DNA methylation level (ML) in non-dividing cells of the apical leaf part goes through modulations coordinated with S phases in leaf meristem cells. Dramatic DNA demethylation found in non-dividing cells (ML decreases nearly two-fold and then it is restored back to normal) is accompanied by DNA reparation. N^6-methyladenine was detected in wheat mitochondrial DNA. A new function of cytokinins (N^6-substituted adenines) is suggested; they do incorporate in DNA and this may result in unmethylated adenine sites formation in it.

CH 213 RECOMBINANT CLONING AND EUKARYOTIC DNA METHYLATION, D.M. Woodcock[1], P.J. Crowther[1], S. Jefferson[1], S.S. Smith[2], M. Noyer-Weidner[3] and M. Graham[4], (1) Peter MacCallum Cancer Institute, Melbourne, Australia, (2) City of Hope, Duarte, California, (3) Max-Planck-Institut fur molekulare Genetik, Berlin, and (4) Calgene Pacific, Melbourne.
Most strains of E.coli used as hosts for recombinant cloning have nucleases which recognise 5-methylcytosine (5mC) in DNA. These are encoded by the Rgl (Mcr) A and B genes. We have quantitated the effect of eukaryotic methylation on the recovery of recombinants in lambda vectors and also in plasmid transformation. Systems were (1) plasmid methylated in vitro with human DNA methyltransferase, (2) plasmid containing human genomic inserts with high or low mC levels (1.8kb and 1.2kb KpnI fragments from the human L1 transposon), and (3) plant genomic libraries in lambda. For plasmid cloning, 3 to 6% mC results in almost 100 fold reduction in transformation efficiency in conventional host strains compared to the most methylation tolerant mcr^- hosts. For phage cloning, the best mcr^- hosts give some 10 fold increment in animal genomic libraries and some 500 fold increase in titre with plant libraries compared to strains such as LE392, C600, and MB406. The level of methylation tolerance varies between different mcr^- strain and cannot be predicted a priori. Optimal strains for plasmid and phage cloning are different. Practical recommendations are made for cloning more heavily methylated genomic regions.

CH 214 CHARACTERISATION OF DNA METHYLASE FROM PEAS.

Mairo H.I. Yesufu, Roger L.P. Adams. Institute of Biochemistry, The University of Glasgow, Glasgow G12 8QQ, Scotland.

A base composition analysis of pea DNA shows 26% of the cytosines are methylated. We have analysed various parts of pea seedlings for DNA methylase activity. High methylase activity has been detected in nuclei from 5 day old pea shoots, (93 units per mg protein). Lower activity is found in root tips, young leaves and subterminal portions of the shoot. Most of the enzyme can be extracted with 0.2M NaCl in tris-HCl buffer, but only after partial digestion of the nuclei with Micrococcal nuclease. The enzyme has been partially purified by anion exchange chromatography on a DEAE column and gel filtration on an FPLC Superose 6 column. The partially purified enzyme has a native molecular weight of 150,000 daltons. On SDS polyacrylamide gel electrophoresis, a major band is seen at 38,000 daltons, which shows crossreactivity to an antibody raised against mouse DNA methylase. The enzyme shows an absolute specificity for cytosine methylation and a strong preference for hemimethylated double stranded DNA substrate.

Structure of DNA and Chromatin

CH 300 METHYLATION OF DNA AT SITES OF HELIX BREATHING, David J. Baker, Julie L.C. Kan, Bruce E. Kaplan, Piotr Dembek, Thomas A. Hardy, and Steven S. Smith, Division of Surgery, City of Hope National Medical Center, Duarte, CA 91010. We have demonstrated that duplex DNA molecules *containing mispaired bases are actively methylated* by the human DNA (cytosine-5)methyltransferase. Using synthetic oligonucleotides (30 bp in length), we have studied the influence of isolated mispairs (dG·dT, dC·dC and dA·dC) and adjacent d(pAC)·d(pCC) mispairs on DNA structure (electrophoretic mobility, restriction enzyme cleavage patterning, and thermal stability), and on DNA *methyltransferase substrate activity* (initial velocity and patterning of applied DNA methylation). Rates of methylation are inversely correllated with measured T_m values for duplex molecules. The measured *de novo* methylation rates are directly correlated with the ability of the structural perturbation associated with the mispair to induce helix breathing in the vicinity of a CG site.

Our results suggest that the stacking energy of the cytosine ring presents a significant energy barrier to its enzymatic methylation. This energy barrier is predicted to be reduced and *de novo* methylation enhanced by: 1.) The formation of secondary structures in DNA (e.g. mismatched base pairs, cruciform structures, or strand exchange intermediates) or 2.) The binding of sequence specific proteins that induce local helix breathing.

CH 301 THE ROLE OF DNA METHYLATION IN A RETROVIRUS-INDUCED MUTATION OF THE MOUSE COLLAGEN I GENE, Michael Breindl, Hedy Chan, Stefan Hartung, Department of Biology, San Diego State University, San Diego, CA 92182. Mov 13 mice carry a Moloney murine leukemia proviral copy in the first intron of the alpha 1(I) collagen (COL1A1) gene. Provirus insertion interferes with the developmentally regulated hypomethylation of the gene and prevents the induction of an active chromatin structure, inactivating the gene on the level of transcriptional initiation. We show here that treatment of Mov13 fibroblasts with 5-aza-cytidine results in demethylation of the mutant COL1A1 promoter. Mov13 fibroblast clones were isolated in which the COL1A1 promoter shows a hypomethylation pattern indistiguishable of that of collagen-producing wild-type fibroblasts. In RNase protection assays the COL1A1 promoter in these clones showed no transcriptional activity, indicating that demethylation is not sufficient for gene activity. *X.laevis* oocyte injection experiments with cloned COL1A1 constructs derived from both the wild-type and Mov13 allele showed that the COL1A1 promoter was transcriptionally active in all constructs independent of whether proviral sequences were present or not. This indicates that the transcriptional inactivity of the COL1A1 promoter in the demethylated Mov13 fibroblasts is not an inevitable consequence of the presence of the proviral genome but rather reflects a transcriptionally repressed state of the gene which is not reversible by demethylation. Because Mov13 fibroblasts most likely contain the trans-acting factors required for efficient transcription of the COL1A1 gene, our results suggest that additional factors, presumably expressed transiently in earlier stages of development, are necessary for transcriptional derepression of the COL1A1 gene.

CH 302 MODULATION OF DNA METHYLTRANSFERASE ACTIVITY BY PROTEIN COMPONENTS OF EUKARYOTIC CHROMATIN, Paola Calafa, Anna Reale, Matilde Rispoli, Valentina Quaresima and Roberto Strom, Departments of Biochemical Sciences and of Human Biopathology, University of Rome "La Sapienza", Rome, Italy.

In vitro assays of endogenous DNA-methyltransferase in eukaryotic (human placenta) chromatin usually show low levels of activity, despite the presence of sizeable amounts of an easily extractable form of the enzyme. Conversely, after salt-induced dissociation of the loosely-bound chromatin proteins, a remaining tightly-bound form of DNA-methyltransferase can be evidenced. It could moreover be shown that enzymic methylation of DNA is favored if DNA is within the "stripped chromatin" context (or, even better, if it belongs to a chromatin subfraction with a high content of tightly-bound proteins).

As in vivo counterpart of this finding, we found very high levels of 5-methylcytosine in the DNA of short segments which, being presumably protected by their association to tightly-bound proteins, resisted extensive digestion by DNAaseI and could therefore be isolated from chromatin loops.

Among chromatin proteins, the histone components exerted a potent inhibitory effect on DNA methylation in an in vitro reconstituted system, while the "high mobility group" proteins HMG 14 and 17 caused a less marked inhibition if added in a renatured state. Addition of renatured cationic tightly-bound protein components (in particular, though maybe not exclusively, of a 50 kDa glycoprotein present in a DNAaseI-resistant subfraction of chromatin matrix) caused instead a significant stimulation of the activity of placental DNA-methyltransferase.

CH 303 CRYSTALLOGRAPHIC STUDIES OF METHYLATED OLIGONUCLEOTIDES: C.A. Frederick, A.H.-J. Wang, G.J. Quigley, and A. Rich., Dept. of Biology, M.I.T., Cambridge, MA 02139

We have solved the three dimensional structures of both a methylated A-DNA octamer (GGm^{5}CCGGCC) and a B-DNA dodecamer (CGCGAm^{6}ATTCGCG). Both structures show stronge similarities to their non-methylated counterparts with small conformational changes resulting from the addition of the methyl groups. In the octamer structure, the characteristic A-DNA stacking interactions which have been observed in other GpG (CpC) rich sequences are maintained. In addition, the presence of the C5 methyl group of cytosine in the major groove causes a local disruption of the hydration structure, while providing hydrophobic interactions which further stabilize the DNA conformation. Comparison with the unmethylated form also shows a slight increase in the width of the major groove. This change in groove width is analogous to what we have observed from the structure of a methylated B-DNA dodecamer containing the EcoRI recognition sequence. Here methylation of the N6 positions of the proper adenines results in a DNA molecule which is resistant to endonuclease cleavage. Variabilities in backbone torsion angles appear to reflect the flexibility of the B-DNA conformation. The structure clearly shows that the presence of these methyl groups disrupts the strict sequence specific hydrogen bonding requirements of the EcoRI endonuclease for formation of a tight recognition complex. Comparison of other restriction modification systems indicates that the relative spatial orientation of the methylation and cleavage sites must play a role in ordering protein secondary structure elements as well as subunit-subunit interactions along the DNA strand.

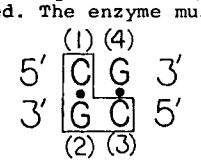
CH 304 HEMIMETHYLATION OF THE HSV-TK DNA IS SUFFICIENT TO BLOCK GENE EXPRESSION, A. Graessmann, M. Graessmann and D. Deobagkar, Institut fuer Molekularbiologie und Biochemie, Freie Universitaet Berlin, Arnimalle 22, D-1000 Berlin 33

To prove whether DNA hemimethylation (methylation of only one DNA strand) is sufficient to block gene expression, the HSV-TK DNA was cloned as single stranded DNA using M13 as the vector. By in vitro DNA replication, all cytosines of the newly synthesized DNA strand were replaced by 5-methyl-cytosine. By autoradiography the hemimethylated DNA was as biologically active as the non-methylated DNA for 24 to 36 hours after microinjection into Tk rat 2 cells. Thereafter the inhibitory effect became demonstrable. Earlier investigations showed that chromatin formation is required to block gene expression of symmetrical methylated DNA (both DNA strands are methylated), so we reconstituted hemimethylated DNA to chromatin in vitro with purified histone octamers. After microinjection we found that the hemimethylated chromatin was inactive. In contrast to experiments with the non-methylated chromatin, thymidine incorporation was not demonstrable regardless of whether the coding or the non-coding DNA strand were methylated. DNA reextraction experiments excluded that this inactivation was caused by the conversion of the hemimethylated DNA into symmetrical methylated DNA directly after microinjection into the recipient cells.

CH 305 IDENTIFICATION OF AN ACTIVE CHROMATIN REGION IN THE PROMOTER OF THE HSV-1 tk GENE. Josef Jiricny and Daniel Schmid, Friedrich Miescher-Institut, PO BOx 2543, CH-4002 Basel, Switzerland.
Our recent studies revealed that methylation of four CpGs within the promoter of the Herpes simplex virus (type 1) thymidine kinase (HSV-1 tk) gene brought about a significant reduction in the transcription of the gene in microinjected Xenopus laevis oocytes (1). Although the methylated cytosines were in or adjacent to the binding sites of the transcription factors Sp-1 and CTF, our data indicated that the binding of these factors to the respective methylated sequences was not significantly lower than to the unmethylated sites. The dramatic downregulation effect seems to have been brought about by methylation anywhere within a region of "active chromatin", the precise site of modification being of lesser improtance. Using a newly-developed method of "methylation-walking", we are currently attempting to define the active chromatin region with high precision, as well as trying to identify the changes in the local chromatin structure, brought about by single-site methylation.

1. Ben-Hattar, J. and Jiricny, J. (1988) Gene, 65, 219-227.

CH 306 A THREE NUCLEOTIDE RULE FOR THE ENZYMATIC METHYLATION OF DNA, Julie L.C. Kan and Steven S. Smith, City of Hope, Duarte, CA 91010. The human DNA(cytosine-5)methyltransferase methylates both unmethylated and hemimethylated CG sites in duplex DNA. In addition, the enzyme has the ability to rapidly methylate duplex CG sites containing one or more mispaired bases, an apurinic site, an alkylated base, or a single-strand overhang. An initial velocity study, and an evaluation of the observed methylation patterns applied to these unusual substrates suggests that the enzyme requires only three out of the four nucleotides in the duplex CG site for activity. In the figure below, if enzymatic methylation occurs at cytosine (3), only the nucleotides shown in the L-shaped box are required. The enzyme must interact with cytosine (1) and its hydrogen bonded guanine (2) as it methylates cytosine (3). Guanine (4) is not required and can in fact be missing, alkylated, or replaced with any base. Even when the structure of a DNA substrate is not well understood, the application of this "L-Test for Methylation" is useful in predicting the methylation pattern applied in vitro. The ability of the enzyme to methylate these substrates according to this rule suggests additional functions for DNA methylation in recombination and repair.



CH 307 COMPLEX ADENINE METHYLATION PATTERNS IN THE NUCLEAR DNA OF TETRAHYMENA, Kathleen M. Karrer and Elizabeth E. Capowski, Department of Biology, Brandeis University, Waltham, MA 02254.
The macronucleus of the ciliated protozoan, Tetrahymena, contains 45 copies of the genome. Approximately 0.8% of the adenine in mac DNA is methylated to N^6MeA. There are two classes of methylated sites: Class I sites are methylated in >90% of the molecules, Class II sites are methylated in a percentage of the molecules which is characteristic of the site and conserved across strains. Class II sites are observed in DNA from clonal cell lines; thus some molecules are methylated and others are unmethylated at a given site within a single macronucleus. This pattern is maintained despite the lack of functional centromeres in the Tetrahymena mac. That is, the vegetative progeny of heterozygous cells eventually become pure for one allele or the other due to random distribution of alleles during vegetative fission. Nonetheless, partially methylated sites are maintained. Thus a semiconservative model for maintenance of methylation can not account for patterns of methylation in Tetrahymena. Supported by N.I.H. grant GM 39890.

CH 308 SEQUENCE SPECIFICITY OF PLACENTAL DNA METHYLASE FOR GC BOX ELEMENTS AND EFFECT OF NEIGHBORING SEQUENCE CONTEXT. Mack J. Rachal, Joel R. Serface, Heahyun Yoo and Jean-Numa Lapeyre. Department of Experimental Pathology, University of Texas M. D. Anderson Cancer Center, Houston, TX 77030. Oligonucleotide sequences bearing CpG methyl-acceptor sites in GC box sequence motifs and HpaII sites were methylated in vitro with human placental DNA methyltransferase and used to monitor sequence-specificity and to establish relative substrate acceptor activity. Using ligated oligomers of basic GC box consensus sequence, the minimum cut-off length for extensive methylation by the placental enzyme began with oligos of 20 nucleotides in length. Enhanced methyl transfer was observed for duplex over single-stranded oligonucleotides. The C-rich strand was a better substrate when single-stranded than the G-rich strand; however, in duplex configuration, it was a poorer substrate. When in a hemimethylated configuration, it served as a better director for methylation of the G-rich strand. Notwithstanding neighboring sequence environment or strand position, hemimethylated GC box CpG sites displayed order of magnitude enhancement on the homologous, unmethylated site. Fluorographic analysis on sequencing gels revealed that there were strand-related preferences for methylation of particular CpG sites depending on the neighboring sequence context, which suggests the presence of a kinetic preference for certain hemimethylated sites, and de novo sites over others.

CH 309 **PROBELESS METHOD FOR DETECTING METHYLATION CHANGES BETWEEN GENOMIC DNA SAMPLES OF DIFFERENT EUKARYOTIC CELLS**, Gerard F. LeBlond and Paul O. P. Ts'o, Division of Biophysics, School of Hygiene and Public Health, The Johns Hopkins University, 615 N. Wolfe Street, Baltimore, MD 21205.

A generalized method for examining and comparing the methylation states of restriction enzyme generated DNA fragments from eukaryotic cell genomes has been developed. The technique can detect differences in methylation states between DNA samples from two different sources without the need for specific gene probes. Our procedure is flexible enough in design so that most restriction enzymes that recognize a cytosine in their recognition sequences, and which are sensitive to modified cytosine bases, can be used. This permits us to search genomes for differences in methylation patterns involving not only some of the 5'-CpG-3' dinucleotide sequences (as is the case when using the Hpa II and Msp I isoschizomeric pair) but also involving 5'-CpG-3', 5'-CpC-3', 5'-CpT-3', and 5'-CpA-3' dinucleotide sequences as well. We present data that support these claims using DNA obtained from Syrian hamster (Mesocricetus auratus) tissue samples.

CH 310 METHYLATION-FREE ISLANDS IN THE GENOME OF TRANSGENIC MICE. Hans Prydz and Anne-Brit Kolstø, Research Institute for Internal Medicine and Department of Microbiology, School of Pharmacy, University of Oslo, Oslo, Norway
The dinucleotide CpG appears in the mammalian genome at about one-fifth of the expected frequency, but accounts for almost all of the methylcytosine present. Between 60% and 90% of CpGs are methylated at the 5-position on the pyrimidine ring. Certain restriction endonucleases are inhibited when this doublet is methylated. The bulk of mammalian DNA is highly methylated, but about 1% of the genome is frequently cut by the methylation-sensitive enzymes HhaI and HpaII. These unmethylated sites are found in about 30 000 clusters of size 0.3-3 kb, designated "methylation-free islands" (MFIs). Several genes have been reported to have such MFIs at their 5'-end. They frequently include the first exon as well as upstream sequences. We have established lines of mice transgenic for various CpG rich and methylation-free islands. Based on these data the fidelity of the methylation process will be discussed.

DNA Methylation in Eukaryotes - Gene Expression, Differentiation and Tumorigenesis

CH 500 A MICROASSAY FOR DNA METHYLASE AND ITS APPLICATION TO EARLY MOUSE EMBRYOS, Roger L.P.Adams, Angela Rinaldi and Marilyn Monk, Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ and MRC Mammalian Development Unit, 4 Stephenson Way, London NW1 2HE, UK.

De novo methylation of injected DNA is known to occur readily only in pre-implantation embryos, yet the level of DNA methylation is low in early embryonic tissue. Because of the limited amount of material available it was necessary to develop a microassay in order to measure DNA methylase activity in early mouse embryos. This was achieved by using high specific activity ^{3}H AdoMet and poly[dI-dC]·poly[dI-dC] as substrate which enabled detection of enzymic activity in as few as 50 mouse L929 cells. Extremely high DNA methylase activity is found in 1 cell eggs and the activity falls rapidly on a cellular basis so that blastocysts have activity similar to that present in cultured cells.

CH 501 PROLACTIN-DEFICIENT GH_3 CELLS ARE FULLY COMPETENT TO INITIATE TRANSCRIPTION FROM THE PRL PROMOTER. Todd E. Arnold and Robert Ivarie, Department of Genetics, University of Georgia, Athens, Georgia 30602.

GH_3 rat pituitary tumor cells express both growth hormone (GH) and prolactin (PRL). EMS induces PRL-deficient lines at high frequency (~10%) that revert at ~50% by 5-azacytidine treatment. Deficient cells still express GH but have a > 50-fold loss of prolactin gene transcription (1-3). We proposed that EMS promoted enzymatic methylation of the PRL gene at CpG site(s) either in the PRL gene itself (*cis* model) or in a target gene encoding a PRL-specific transactivating factor (*trans* model) To test both possibilities, the prolactin promoter containing 5' proximal sequences from -616/+38 was linked to the bacterial CAT gene (pPRL653CAT) and used to transfect wild-type, PRL- deficient, and revertant GH_3 cells as well as two nonpituitary cell types (rat-2 and HTC). pPRL653CAT was efficiently expressed in all three GH_3 cell lines but not in rat-2 firbroblasts and HTC hepatoma cells. Thus, the proximal promoter exhibited cell type-specific expression but no difference in expression was seen between wild-type and PRL-deficient cells. Since PRL-deficient cells were fully competent in driving expression from the proximal promoter region, a plasmid (pPRLCAT) containing additional 5' sequences (-1957/+38) was used (4); it increased CAT expression ~10-fold in both wild-type and PRL-deficient cells. These observations imply that PRL-deficient cells are not defective in a transactivating factor functioning on these PRL promoter fragments (*trans* model). Rather, inefficient use of the PRL-promoter in the variant cells may reflect an increased methylation state of the PRL gene its elf (*cis* model).

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CH 502 METHYLATION AS A MODULATOR OF EXPRESSION OF HUMAN IMMUNODEFICIENCY VIRUS, Daniel P. Bednarik, Joseph D. Mosca and Paula M. Pitha, The Johns Hopkins University Oncology Center, Baltimore, MD 21205

In humans, HIV infection is characterized by a period of latency followed by progression to AIDS or AIDS-related complex. A3.01 CEM T-cell lines and SW480 colonic cell lines containing stably integrated HIV LTR-CAT (pU3RIIICAT) or proviral DNA did not express CAT enzyme or viral products unless induced by 5-azacytidine, HSV-1 products (IE110), or ultraviolet light. In each case, dramatic changes in the viral LTR methylation profile occurred as determined by Southern hybridization analysis. In vitro methylation of -CpG-LTR sequences by HpaII methylase resulted and subsequent transfection, in cis-level inactivation of the LTR, a process which was consistently overcome in trans by the viral trans-activator tat. In vitro methylation of the viral LTR directing tat expression (pIIIextatIII) incapacitated tat production, and preserved the latent state of cell lines harboring HIV LTR-CAT sequences (A3N9-2) or proviral DNA (A2NHIV, SWNL4). The effect of inducers was not mediated by the NF-$\kappa\beta$ factor, since latent cell lines containing site-specific mutations in the NF-$\kappa\beta$ binding region demonstrated inducibility by these agents identical to the native LTR. We propose a cellular role in the maintenance of the latent state via hypermethylation of LTR sequences.

CH 503 METHYLATION OF A SINGLE CpG IN A MODIFIED HSV-1 tk PROMOTER REDUCES tk TRANSCRIPTION IN VIVO. Jean Benhattar (+*) and Josef Jiricny(+), (+) Friedrich Miescher-Institut, PO Box 2543, CH-4002 Basel, Switzerland, (*) Swiss Institute for Experimental Cancer Research, CH-1066 Epalinges, Switzerland.

Transcriptional activation of the Herpes simplex virus (type 1) thymidine kinase (HSV-1 tk) gene is mediated via the binding of the Sp-1 and CTF proteins immediately upstream from its TATA box. Using a linker-scanning mutant construct, in which the binding sites for these two transcription factors were contained within a 33 bp fragment, flanked by unique EcoRI and BamHI sites, we were able to study the influence of cytosine methylation on the transcription of the tk gene in vivo. We substituted the native 33-mer with a series of synthetic duplexes, in which one of the total of four CpGs was methylated. Microinjection of the resulting constructs into Xenopus laevis oocytes, followed by primer extension analysis of the tk mRNA, showed that methylation of any one of the CpGs resulted in an approximately 20-fold downregulation of specific tk transcription (1). Gel shift experiments with the methylated oligos alone indicated that this effect was not brought about exclusively by the lowering of the affinity of the transcription factors for the methylated DNA sequences, but that other, as yet unidentified, factors were also involved.

1. Benhattar, J. and Jiricny, J. (1988) Gene, 65, 219-227.

CH 504 B-CELL SPECIFIC K-CHAIN GENE METHYLATION. M. Lichtenstein, H. Cedar and Y. Bergman. The Hebrew University - Hadassah Med. Sch. Jerusalem 91010, Israel.

The regulation of immunoglobulin k-chain gene expression during B-cell differentiation is an attractive system to use in studying the mechanism and the role of DNA methylation. We methylated the k-chain gene in vitro at the HpaII and the HhaI sites using bacterial methylases. The methylated and unmethylated k-chain genes were introduced separately into the following cell lines: Ltk-, somatic cell hybrids between myeloma and Ltk-, a pre B-cell line (70Z/3), a B-cell line (A20) and into two plasmacytomas, S194 and S107. Southern blot analyses of the exogenous unmethylated and methylated k genes in Ltk-cells and in the hybrids, demonstrate that the methylation status of the genes was not changed by the cellular environment into which they were introduced. In contrast, the methylated k-chain gene introduced into the B and plasmacytoma cell lines (A20 and S194) underwent demethylation. The ability to actively demethylate an exogenous k-chain construct appears, however, to be not only lineage specific but also cell-stage specific, since demethylation did not occur in pre B-cell line, where the endogenous gene is inactive and methylated. Pre B-cells and the plasmacytoma cell line S107, which lacks the critical NF-kB transcription factor, de novo methylated the k-chain genes. Thus, the various B-cell lines representing different stages of B-cell development have the potential to mimic the in vivo process of k-chain gene methylation. We will use this biological system to study questions concerning the biology and biochemistry of k-chain gene methylation.

CH 505 THE EFFECT OF DNA METHYLATION ON THE BINDING OF THE TRANSACTING FACTOR Sp1 TO DNA, Margaret Bryans and Roger L.P.Adams, Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K.

It is generally accepted that there exists an inverse relationship between DNA methylation and transcription in a large number of eukaryotic genes. Using SV40 as a model system we observed that in vitro methylation of the early promoter, by mouse DNA methylase, led to reduced expression of a linked chloramphenicol acetyl transferase gene. This effect may be due to altered protein-DNA interactions in the promoter. It is possible that the presence of 5-methyl cytosine in the major groove of the DNA helix may inhibit the binding of essential transcription factors, such as Sp1, hence limiting transcription. There are six Sp1 recognition sites in the SV40 promoter, known as the GC boxes, with varying affinity for the protein. Labelled oligonucleotides containing 1 to 3 GC boxes in either the methylated or unmethylated state show equal affinity for SP1 in gel retardation assays and compete equally well with labelled fragments containing the SV40 promoter. This indicates that cytosine methylation at these sites has no effect on binding and suggests that DNA methylation alters the level of transcription by an alternative mechanism.

CH 506 ALTERNATIVE SITE-SPECIFIC METHYLATION REGULATES TWO HOMOLOGOUS CYTOCHROME P-450 GENES IN MICE, Barbara A. Burkhart and Masahiko Negishi, Pharmacogenetics Section, Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709

The cytochrome P-450 genes 15αOH-1 and 15αOH-2, which encode steroid 15α-hydroxylase and coumarin 7-hydroxylase respectively, share over 96% sequence homology including the 5' and 3' flanking regions and introns. The two genes are expressed differently, in different tissues, by sex, by various inducers and at distinct developmental stages. We have identified two HpaII/MspI sites which are demethylated in differential tissue specific and developmental patterns. The first site occurs 20bp upstream of the TATAA and 50bp downstream of the CAAT box in 15αOH-1, but is absent in 15αOH-2. Complete demethylation at this site is correlated with high 15α-hydroxylase activity in adult livers. Low 15α-hydroxylase activity in other tissues is correlated with partial demethylation at this site. The other specific HpaII/MspI site, located in the sixth intron, is demethylated developmentally in 15αOH-2 but not in 15αOH-1. The 15αOH-2 demethylation occurs in the liver and lung, corresponding to tissues with high coumarin 7-hydroxylase activity. Despite tissue-, site-, and gene-specificity, methylation was neither sex-specific nor inhibitory for low levels of activity. This indicates that demethylation at these sites is not a primary regulator but is necessary for higher levels of expression of these genes.

CH 507 REPLICATION AND METHYLATION IN STRUCTURAL MAINTENANCE OF *PHYSARUM* RIBOSOMAL DNA

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In the acellular slime mold *Physarum polycephalum*, most rDNA is in 60kbp palindromes each with four symmetrically arranged replication origins flanked by ribosomal RNA genes. A particular sequence, the putative replication origin, is repeated at the approximate position of each origin (1-3). The putative replication origins and adjacent inverted repeats are heavily methylated (as 5-methyldeoxycytidine) (2-5). Typically only one of four origins is active per replication cycle (1). These 60kbp rDNAs are asymmetrically methylated and particularly hypomethylated at one of their four putative replication origins (5). We find larger and smaller rDNAs with six and two putative replication origins respectively. These molecules could arise from recombination of 60kbp rDNAs. The methylation level appears similar on the putative replication origins of all these rDNAs. The same methylation level on origins would make two or more hypomethylated origins likely in molecules with six origins and no hypomethylated origins likely in molecules with two origins. We propose a model where hypomethylation is a determinant of origin activity and serves to maintain most rDNA as 60kbp molecules with one of four replication origins active.

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CH 508 AN ANIMAL VIRUS-INDUCED DNA-METHYLTRANSFERASE, Karim Essani, Rakesh Goorha, and Allan Granoff, Department of Virology and Molecular Biology, St. Jude Children's Research Hospital, Memphis, TN 38101

The DNA genome of frog virus 3 (FV3), an iridovirus, is highly methylated; more than 20% of cytosine residues are methylated at the 5-carbon position. Methylation of the viral DNA occurs in the cytoplasm of infected cells by an FV3-specified DNA-methyltransferase (DNA-mt). To determine the role of this enzyme in virus replication and gene expression, we have isolated a number of FV3 mutants defective in the expression of DNA-mt activity. Combined genetic and biochemical analyses of one of the mutants have revealed a 26K polypeptide associated with DNA-mt activity. Attempts to purify the 26K polypeptide have resulted in co-purification of two other polypeptides, 30K and 18K, along with the 26K one. Additional experiments directed toward identifying DNA-mt activity with the individual polypeptides, together with reconstitution experiments, have indicated that at least two polypeptides (26K and 18K) are required for functional DNA-mt activity. These data support the conclusion that FV3-induced DNA-mt, unlike any known eukaryotic DNA-mt, resides in a complex of at least two polypeptides.

CH 509 INTRODUCTION AND EXPRESSION OF HhaI DNA METHYLTRANSFERASE IN EUKARYOTIC CELLS, J. Herman[1], B. Nelkin[1], M. Mabry[1], G. Wilson[2] and S. Baylin[1]. [1]Johns Hopkins Oncology Center, Baltimore, MD 21205; [2]New England Biolabs, Beverly, MA 01915. DNA methylation abnormalities, which include both widespread hypomethylation and regional hypermethylation, have been reported for many malignancies. In culture, tumor cells often have increased DNA methyltransferase activity with unknown consequences. To assess functional consequences of increased cytosine methylation in eukaryotic cells, we are using a constitutively expressed prokaryotic DNA methyltransferase enzyme in murine fibroblasts. We inserted the cloned DNA sequence for the bacterial DNA methyltransferase M.HhaI (methylated sequence G^{m}CGC) into the retroviral expression vector pZIPneo SV(X). Abundant G418 resistant colonies were produced in PA 317 amphotrophic packaging cells after infection with either the pZIPneo SV(X) vector alone or M.HhaI inserted in the antisense direction. However, insertion of M.HhaI in the sense orientation resulted in a total of two G418 resistant clones in 5 independent experiments. Southern and Northern blots probed with M.HhaI sequences demonstrated integration and expression. Each M.HhaI clone in the sense orientation was tumorigenic in nude mice, and one of the clones was morphologically distinct from control PA 317 cells. Our data suggest that constitutive expression of M.HhaI is often lethal to eukaryotic cells in culture, and may cause transformation of surviving cells.

CH 510 CpG ISLANDS AND EXTENT OF CpG METHYLATION IN THE PROMOTER REGIONS OF THREE HUMAN DNA REPAIR GENES, Mark A. MacInnes and John S. Mudgett, Genetics Group, LS-3, M886, Los Alamos National Laboratory, Los Alamos, NM 87545 The fundamental housekeeping roles of DNA repair genes suggest that they may exhibit DNA regulatory structures typical of genes expressed in most tissues, as well as other regulatory elements directly related to their repair functions. To characterize the extent and localization of the putative regulatory regions, we have identified promoter-associated structures in the human excision repair genes ERCC1 and ERCC2 (both essential for pyrimidine dimer DNA repair) and in XRCC1, a gene important for repair of radiation-induced single strand breaks. The three genes were previously isolated in other laboratories as functional cosmid clones. Each gene has a 5' end cluster of restriction endonuclease sites indicative of CpG islands. The ERCC1 CpG island is located within the first intron (397 bp sequence kindly provided by Dr. Marcel van Duin, pers. comm.). That CpG-rich segment contains 7 clustered GC box structures. The methylation pattern of 5' region restriction sites is being assessed currently to determine whether low level expression of repair genes correlates with under methylated regulatory regions. Supported by the U.S. DOE. Contract W-7405-ENG-36.

CH 511 METHYLATION MAP OF THE 5' FLANKING REGION OF THE HUMAN c-H-RAS GENE IN NORMAL LYMPHOCYTES BY GENOMIC SEQUENCING TECHNIQUE. Antonio Milici, Jean-Numa Lapeyre, Frederick F. Becker, Section of Experimental Pathology, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030. A large number of studies indicate that DNA methylation plays a basic role in gene regulation in higher eukaryotes. Substantial evidence links aberration in the methylation process to neoplastic transformation. Techniques employed thus far can only detect a small percentage (10%) of methylation sites present in a gene. We have used the genomic sequencing technique to map the methylation sites in the 5' flanking region of the human c-H-ras gene in normal lymphocytes. A large number of CpG boxes (potential methylation sites) are present in this region. DNA obtained from human peripheral lymphocytes was digested with Bam HI restriction enzyme, treated with Maxam and Gilbert sequencing reactions and electrophoresed in an 8% PAGE. DNA was then transferred to a nylon membrane and UV treated. A 198 bp BamHI-XhoI fragment spanning the 5' terminus of c-H-ras promoter region was subcloned into a M13 mp 19 vector, labelled with [alpha ^{32}P]-dCTP, and hybridized to the genomic DNA. We have now mapped the c-H-ras 5' flanking region from position -1588 to position -1498. Our results suggest that several, previously undetected, m^{5}C are present in this region of the c-H-ras protooncogene.

CH 512 CONTROL OF EXPRESSION AND INTRAGENIC METHYLATION OF THE MOUSE α-FETOPROTEIN GENE. K. Opdecamp, C. Szpirer, M. Rivière, M. Molné, & J. Szpirer. Université Libre de Bruxelles, Département de Biologie Moléculaire, B1640 Rhode-St-Genèse, Belgium.

We addressed the question of the possible role of DNA methylation in the control of the mouse AFP gene expression. The methylation pattern of the upstream region, and of the 5' end of the intragenic region was studied in expressing cells (fetal liver and hepatoma cell line), in non producing hepatic cells (adult liver) and in fibroblasts, using the 2 isoschizomers MspI and HpaII. A correlation was found between expression and unmethylation at a single MspI site located in the first intron of the gene (M_0). This site is partially methylated in fetal liver, unmethylated in hepatoma cells and extensively methylated in adult liver and fibroblasts. This result suggests that *de novo* methylation at this intragenic site may participate in the shut-off of AFP synthesis during liver developmemt.

To further analyze this possibility, we studied the expression of cloned AFP sequences transfected in hepatoma cells. A fragment of a genomic clone (position - 1kb to + 0.6 kb), which contains the first intron, was linked upstream the bacterial reporter gene CAT. This construction directs high levels of CAT synthesis when it is introduced in the AFP-producing hepatoma cell line HepG2. The effect of *in vitro* methylation of this construction on CAT expression will be reported.

CH 513 DNA METHYLATION IN MYOBLAST DIFFERENTIATION. Franco Palitti, Daniela Carotti, Sigfrido Scarpa and Roberto Strom, Departments of Biochemical Sciences and Human Biopathology, University of Rome "La Sapienza", Rome, Italy.

Cellular differentiation has been correlated to variations in site-specific or genome-wide DNA methylation. A transient hypomethylation, probably due to active removal of methyl groups, followed by remethylation, has recently been shown to take place, in Friend erythroleukemia cells, during the first stages of differentiation. On the other hand, reports on a differentiating human melanoma cell line have shown no direct correlation between changes in DNA methylation levels and phenotypic differentiation. We have investigated DNA methylation and DNA methyltransferase levels in strictly diploid L5 myoblast sublines, which, under controlled conditions (notably when the concentration of foetal calf serum is lowered from 10% to 1% and/or when AdoMet metabolism is perturbed by addition of 3-deazaadenosine), can be prompted, in vitro, to undergo differentiation to myofibers. Overall DNA methylation levels were evaluated at various times after induction of the differentiation process in cultured cells that had been previously labeled with ^{14}C-uridine, the extent of cytosine methylation in parental DNA being measured by HPLC. No significant variation of the methylcytosine/cytosine ratio could sofar be evidenced under any experimental conditions. In the myoblast system, therefore, active removal of pre-existing 5-methylcytosine appears not to be a major event during in vitro differentiation.

CH 514 DNA METHYLATION OF CIS-REGULATORY ELEMENTS IN VITRO MODULATES C-HA-RAS PROMOTER ACTIVITY. Mack Rachal, Heahyun Yoo, Frederick F. Becker, and Jean-Numa Lapeyre. Department of Experimental Pathology, University of Texas M. D. Anderson Cancer Center, Houston, TX 77030.

The effects of DNA methylation on H-ras promoter activity was assessed using a transient expression assay employing pH-rasCAT (H-ras promoter linked to the chloramphenicol acetyltransferase reporter gene). Site-specific methylation of promoter elements in pH-rasCAT with HhaI (CG*GG) and HpaII (C*CGG) bacterial modification enzymes decreased CAT activity by 70-80% compared to unmethylated controls. Use of a human placental methyltransferase which methylates asymmetric CpG dinucleotide sequences, including GC boxes, inhibited CAT activity by 95%. Furthermore, the inhibition of H-ras promoter activity was not attributed to methylation-induced differences in DNA uptake or stability in the cell, topological form of the plasmid, or methylation effects in nonpromoter regions. Preliminary gel retardation assays indicate that DNA methylation of a 360 bp H-ras promoter fragment induces a local change in DNA conformation. Mechanistically, changes in local promoter conformation might affect the interactions between DNA binding proteins and cis-acting target sequences. Owing to higher binding affinities, these transcription factors would preferentially bind to unmethylated cis-regulatory sequences. In this context, DNA methylation might serve as an on/off transcription switch.

CH 515 RAPID AND SENSITIVE FLUOROIMMUNOASSAY OF DNA METHYLATION, Simo J.Rasi, Esko Suvanto,Leena M.Vilpo and Juhani A.Vilpo.Farmos Diagnostica Research Laboratory, Oulunsalo; Laboratory, Oulu Deaconess Institute; Laboratory of Molecular Hematology, Biocenter, and Department of Clinical Chemistry, University of Oulu, Finland.

We have developed a novel technique for the quantitation of 5-methyl-2'-deoxycytidine in DNA hydrolysates. This Delfia™-5MedCyd assay is a solid phase fluoroimmunoassay, based on competition between europium-labelled 5MeCyd and sample 5MedCyd for polyclonal rabbit anti-5MedCyd antibodies. Standard and sample preparations containing 5MedCyd inhibit binding of the europium-labelled 5MeCyd to the antibody. A second antibody, directed against rabbit IgG, is coated on a solid phase, and binds the IgG-5MeCyd-europium complex, giving rapid and complete separation of antibody-bound and free antigen. The assay requires only one incubation step. Enhancement Solution dissociates europium ions from the labelled 5MeCyd into solution, where they form highly fluorescent chelates. Fluorescence in the microtitration strip wells is measured in a time-resolved fluorometer (counting time 1 sec/sample), the fluorescence from each sample being inversely proportional to the concentration of 5MedCyd. The measuring range is from 3.7 to 2500 pmol of 5MedCyd per well. The mean intra- and interassay precisions were 3.4% and 8.5 % respectively. In comparison of Delfia™-5MedCyd to HPLC, we obtained good correlation. The mean recovery percentage, calculated by comparing the amount of 5MedCyd found to the amount added, was 94 %. We also report our results on DNA 5MeCyt levels in breast tumor and uterus cells in rats treated with antiestrogens in vivo.

CH 516 THE SV40 ENHANCER DOES NOT OVERCOME THE TRANSCRIPTION INHIBITION BY EUKARYOTIC DNA METHYLATION, Dietrich Simon, Herbert Wagner, Hans Kröger and Frank Götz, Robert Koch-Institut, Nordufer 20, 1000 Berlin 65, GFR.

In the adenovirus system promoter inactivation by DNA methylation has been reported to be reverted by insertion of a cytomegalovirus enhancer. We found that SV40 and Polyoma early genes which are under the direction of strong enhancers cannot be silenced in eukaryotic cells by DNA methylation. This could have been due to the overriding effect of the enhancer.
We show here that in Xenopus oocytes, too, the early SV40 genes under their own enhancer are only slightly inhibited by in vitro methylation with eukaryotic DNA methyltransferase. In contrast, the late SV40 genes under the same SV40 enhancer in the reverse orientation are completely inactivated. Methylation of the SV40 regulatory region and of the 5'-region of the late genes is necessary to achieve the inhibition. To evaluate whether the SV40 enhancer prevented the shut-off of the early genes, two copies of the 72 bp SV40 enhancer were inserted into the regulatory region of the HSV TK gene. The inactivation of the TK gene in oocytes by eukaryotic DNA methylation was not influenced by the SV40 enhancer, neither in the late nor in the early orientation. We propose that only the transcription inhibition by very few methylgroops as introduced by Hpa II methylation in the adenovirus model can be overcome by enhancers.

CH 517 PROGRESSION OF DE NOVO METHYLATION IN A HSV THYMIDINE KINASE GENE INTEGRATED IN A HUMAN CELL LINE. Judith G. Tasseron de Jong, Hans den Dulk and Micheline Giphart-Gassler. Laboratory of Molecular Genetics, Leiden University, Leiden, The Netherlands.

We have studied de novo methylation of a single copy thymidine kinase (tk) gene integrated in a human cell line with methylation sensitive restriction enzymes. Limited de novo methylation was involved in the loss of the expression of the tk gene. DNA of TK$^-$ clones isolated initially at low frequency from the TK$^+$ cell line was methylated at one or both CpG's of the sequence CGAATTCg located in the tk promoter region, whereas all HpaII sites in the tk coding region remained unmethylated. After treatment of such clones with 5-azacytidine TK$^+$ clones were obtained by partial demethylation. From these reactivated TK$^+$ clones TK$^-$ variants were now isolated with an up to 1000 fold increased frequency by a second round of DNA methylation. Southern analysis of HpaII and MspI digests of genomic DNA of these clones revealed a gradient of methylated HpaII sites from the tk promoter region into the tk encoding sequence. The length of the methylated stretch of DNA was variable but was always contiguous, suggesting that the de novo methylation is not random but occurs in patches. The data indicate that maintenance methylation may lead to de novo methylation of unmethylated stretches of DNA.

CH 518 A CONVENIENT ASSAY FOR DNA METHYLTRANSFERASE, E. P. Whitehead, B. Taddeo, E. Stampeggioni, F. Palitti and D. Carotti, Dept. of Biochemical Sciences, Rome University, Rome 00185, Italy
Assaying DNA methylase by measuring transfer of tritium from the 5 position of DNA cytosine to water rather than labelled methyl transfer from S-adenosyl methionine to DNA cuts post-incubation procedures from hours to minutes. The methodology and validating data obtained in our laboratory will be presented.

CH 519 **CHEMOTHERAPY-INDUCED CHANGES IN DNA METHYLATION AS A POSSIBLE MECHANISM FOR GENERATING DRUG RESISTANCE** Katharine S. Worton, Robert Kerbel and Irene Andrulis, Mount Sinai Hospital Research Institute, Dept. of Medical Genetics, University of Toronto, 600 University Ave., Toronto, M5G 1X5, Canada. Standard remission induction therapy for childhood acute lymphoblastic leukemia (ALL) includes L-asparaginase, since the leukemic cells lack asparagine synthetase (AS) activity and require exogenous asparagine. A model in which to examine a novel mechanism of drug resistance in ALL is provided by the CHO cell line N3, which lacks AS activity. Expression of AS in CHO cells is associated with hypomethylation in the 5' region of the gene. We have found that activation of AS in concert with hypomethylation may arise spontaneously in the N3 line at a frequency of about 3×10^{-6} or may be induced up to 3400-fold by the hypomethylating drug 5-azacytidine. To investigate the possibility that chemotherapeutic drugs induce similar changes, the asparagine auxotrophic cell line N3 was treated with the chemotherapeutic agents L-asparaginase, vincristine or ara-C or with the mutagen EMS. L-asparaginase and EMS increased the frequency of reversion to asn prototrophy up to 9- and 11-fold respectively. Vincristine had little effect, at most a 2-fold difference. Ara-C, a known hypermethylating drug, depressed the reversion frequency by up to 70%. Asn prototrophy correlated with the demethylation of CpG sites in the 5' region of the AS gene and with the appearance of AS mRNA and protein in revertants.