Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 6299–6303, October 1982
Cell Biology

Expression of transferred thymidine kinase genes is controlled by methylation

(gene transfer/DNA methylation/5-azacytidine/DNA rearrangement)

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Communicated by John W. Littlefield, July 19, 1982

ABSTRACT **Plasmid pTKx-1, containing the herpes simplex virus gene for thymidine kinase (TK) inserted into the *Bam*HI site of plasmid pBR322, was introduced into Ltk$^-$ cells by calcium phosphate precipitation in the absence of carrier DNA. Line 101 is a TK$^+$ derivative of Ltk$^-$ that contains multiple copies of pTKx-1 in a multimeric structure. A derivative of 101 that retained but no longer expressed the herpes simplex TK genes (termed 101BU1) and derivatives of line 101BU1 that reexpressed the genes (termed 101H1, 101HC, and 101HG) were selected. The TK genes in 101BU1 were hypermethylated relative to those in the TK$^+$ parent and derivatives. Growth of 101BU1 in the presence of the methylation inhibitor 5-azacytidine resulted in an average 13-fold increase in the number of TK$^+$ reexpressors. DNA from 101BU1 was inactive in secondary gene transfer, whereas DNA from 101 and from TK$^+$ reexpressors was active. These data support a causative relationship between DNA methylation and decreased gene expression. All TK$^+$ reexpressors examined had DNA rearrangements involving TK DNA.**

The presence of methylated bases in the DNA of both prokaryotes and eukaryotes has been known for some time (1). In mammalian DNA, the only detectable methylated base is 5-methylcytosine (m^5C) (2). From 2% to 7% of cytosine residues are found as 5-methylcytosine, and greater than 90% of those occur in the dinucleotide sequence CpG (3). The distribution of 5-methylcytosine in the DNA is nonrandom, and the patterns of methylation appear to be species specific and tissue specific (1, 4). The pattern of methylation is passed on from a cell to its daughters through the action of one or more "maintenance methylase" enzymes, which act on hemimethylated DNA shortly after replication (5).

Although the precise enzymatic mechanisms of DNA methylation are only beginning to be elucidated, a strong inverse correlation has been established between the amount of DNA methylation and the level of gene expression. Hypomethylation of several genes, including globin (6), ovalbumin (7), ribosomal DNA (8), and metallothionein (9), has been found in tissues in which the genes are expressed, whereas hypermethylation of the same genes or their surrounding DNA has been found in tissues in which they are not expressed. Several investigators have found that endogenous, unexpressed viral genomes often are heavily methylated but that expressed viral DNA is unmethylated (10–14). In the case of adenovirus, portions of the viral DNA can be methylated while other regions remain unmethylated, and the level of methylation is inversely correlated with the level of expression of particular viral genes (15).

Groudine *et al.* (12) demonstrated that inhibition of DNA methylation by growth of cells in the presence of 5-azacytidine (5-azaCyd) resulted in the hypomethylation and transcriptional activation of endogenous viral genomes, and Compere and Palmiter (9) demonstrated that growth of cultured cells in the presence of 5-azaCyd resulted in hypomethylation and activation of the metallothionein-I gene. These studies suggest a causative role for methylation in the reduction of gene expression, but they could not conclusively demonstrate that reexpression of the genes resulted directly from demethylation of gene sequences induced by 5-azaCyd.

We have transferred the herpes simplex virus (HSV) gene for thymidine kinase (TK) into TK-deficient mouse fibroblast Ltk$^-$ cells and have studied the modulation of expression of TK in one cell line, termed 101. Derivatives of 101 were selected that retained but did not express the TK genes; subsequently, derivatives of those were selected in which the genes were reexpressed. The powerful selections for and against the expression of the TK gene, the use of the cloned gene as a nucleic acid probe, and the ability to use cellular DNA in secondary gene transfer experiments allowed precise correlations between gene expression and the biochemical state of the TK DNA. The experiments to be presented here demonstrate that the extent of methylation of the TK DNA is inversely correlated with gene expression, and that growth of TK-deficient derivatives of 101 in the presence of 5-azaCyd resulted in a 6- to 23-fold increase in the number of TK$^+$ derivatives. Furthermore, we show that DNA isolated from 101 and from reexpressing TK$^+$ derivatives was active in secondary gene transfer experiments, whereas DNA isolated from TK-deficient derivatives of 101, in which the TK genes were heavily methylated, was inactive. These data demonstrate that the activation/inactivation of the TK genes is effected directly through a change in the extent of DNA methylation of the TK gene. Finally, we show that DNA rearrangements involving the TK genes were seen in each of three TK$^+$ reexpressors, raising the possibility that gene rearrangement might be a mechanism of demethylation.

MATERIALS AND METHODS

Cell Culture. Cells were maintained in monolayer culture at 37°C under 5% CO_2/95% air in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 5% fetal bovine serum (GIBCO). TK$^+$ cells were maintained in Dulbecco's modified Eagle's medium supplemented with hypoxanthine, aminopterin, and thymidine (HAT medium) (16). DNA-mediated gene transfer was performed as described (17).

5-azaCyd Treatments. 101BU1 (TK$^-$) cells were plated at 0.5–1 × 10^6 cells per 100-mm dish in nonselective medium; 24 hr after plating, the medium was replaced with fresh medium containing 0, 2, 5, or 10 μM 5-azaCyd (Sigma). Cells were

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Abbreviations: TK, thymidine kinase; HSV, herpes simplex virus; 5-azaCyd, 5-azacytidine; HAT, hypoxanthine/aminopterin/thymidine; kb, kilobase(s).

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treated with 5-azaCyd for 3–5 days, rinsed with phosphate-buffered saline, and refed with selective HAT medium. The medium was changed every 2–3 days, and colonies were counted after 14–21 days in selective medium. The cell number at the start of selection in HAT medium was determined in a hemocytometer.

Biochemical Procedures. Plasmid DNA was isolated as described by Guerry *et al.* (18). High molecular weight cellular DNA was isolated as described by Wigler *et al.* (19). Levels of TK enzyme activity were determined by the method of McBride *et al.* (20). Restriction endonuclease digestions were performed as recommended by the suppliers (Bethesda Research Laboratories or New England BioLabs) with 10- to 30-fold excess of enzyme. *Hpa* II digests were performed with a 30- to 50-fold excess of enzyme on independently isolated samples of 101BU1 DNA to ensure complete digestion. Samples of DNA (10 μg) were electrophoresed in 0.7%, 1%, or 1.2% agarose (Bethesda Research Laboratories) containing 40 mM Tris, 1 mM EDTA, and 60 mM NaOAc (pH 7.9). DNA blotting and filter hybridization were performed by the method of Wahl *et al.* (21) as modified by Huttner *et al.* (22). pTKx-1 DNA or the 3.5-kilobase (kb) *Bam*HI fragment of pTKx-1 DNA was nick translated to a specific activity of 0.7–3 $\times$ 10^8 dpm/μg by a modification of the method of Rigby *et al.* (23) as described by Maniatis *et al.* (24).

RESULTS

Cell line 101 arose after treatment of Ltk^- cells with the circular plasmid pTKx-1 (25) in the absence of carrier DNA. Preliminary characterization of several cell lines generated in this way demonstrated that most contained multiple copies of plasmid pTKx-1 in a multimeric structure at one chromosomal locus (17). Southern blot analysis of 101 DNA showed the appearance of one band homologous to the TK probe after *Xba* I digestion, even when the DNA was electrophoresed through 0.7% agarose gels for 18 cm (Fig. 1). *Xba* I does not cleave plasmid pTKx-1 (25), so that the sites that generated the single band must have been in chromosomal DNA flanking the plasmid DNA. Multiple bands were visualized after *Hin*dIII or *Bam*HI digestion, which cleaved pTKx-1 once or twice, respectively (Fig. 1). A predominant band of 7.9 kb (the size of pTKx1) was seen after *Hin*dIII digestion, indicating that the TK sequences were organized in a multimeric structure consisting predominantly of full-length plasmid concatamers. This interpretation was supported by *Bam*HI digestion, which generated predominant bands of 4.3 and 3.5 kb, which correspond to the pBR322 and TK moieties of the plasmid. The presence of additional bands indicates that deleted or rearranged copies (or both) of pTKx-1 also were present.

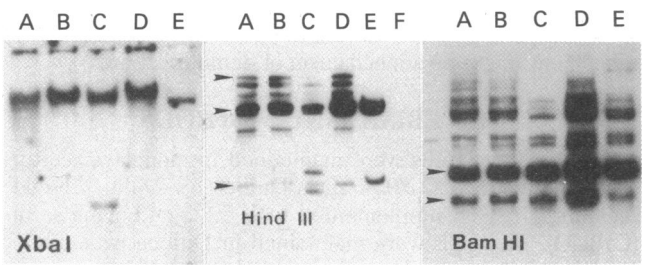

FIG. 1. *Xba* I, *Hin*dIII, and *Bam*HI digests of line 101 and derivatives. DNA of lines 101 (lanes A), 101BU1 (lanes B), 101H1 (lanes C), 101HC (lanes D), 101HG (lanes E), and LTK^- (lane F) were digested with *Xba* I, *Hin*dIII, or *Bam*HI. The DNAs were separated in 0.7% agarose gels, transferred to nitrocellulose, and hybridized with ^{32}P-labeled pTKx-1 as a probe. The band patterns of 101, 101BU1 and the reexpressing cell line 101HC (lanes A, B, and D, respectively) are indistinguishable after *Hin*dIII and *Bam*HI digestion. 101H1 and 101HG reexpressor lines (lanes C and E) are missing bands in both the *Hin*dIII and *Bam*HI digests. The arrows point to bands of 17, 7.9, and 3.5 kb in the *Hin*dIII digests and to bands of 4.3 and 3.5 kb in the *Bam*HI digests. The *Xba* I band is >23 kb.

TK-Deficient Derivatives Retained but Did Not Express the Viral TK Genes. When plated in medium containing 5-bromodeoxyuridine (BrdUrd) to select against expression of TK, derivatives of line 101 were isolated at a frequency of $\approx 10^{-6}$ (not shown). One such derivative, 101BU1, was analyzed in detail. Line 101BU1 had no detectable TK enzymatic activity (not shown) but retained the viral TK genes. Digestion of the DNAs of 101 and 101BU1 with *Xba* I, *Hin*dIII, *Bam*HI (Fig. 1), and *Pvu* II (not shown) failed to detect any difference in the pattern of TK-specific bands between the two lines.

101BU1 cells were replated in HAT medium to select derivatives that reexpressed the TK genes, and HAT^r derivatives were isolated at a frequency of 5×10^{-7}. Two independent reexpressing cell lines, termed 101HI and 101HG, were characterized further. A third TK^+ derivative, 101HC, was isolated after 5-azaCyd treatment of 101BU1 cells.

Methylation of TK DNA Was Inversely Correlated with Expression of the TK Gene. DNAs isolated from lines 101, 101BU1, 101H1, 101HC, and 101HG were each digested with *Msp* I and *Hpa* II, isoschizomers that recognize and cleave the same nucleotide sequence—C-C-G-G (26). Because *Hpa* II fails to cleave the DNA when the internal C is methylated whereas *Msp* I is insensitive to methylation at that site, a comparison of the pattern of bands visualized on Southern blots after digestion with the two enzymes provides an indication of the extent of DNA methylation. Plasmid pTKx-1 contains more than 40 recognition sites for *Msp* I, generating fragments ranging in size from 9 to 600 base pairs. Digestion of 101 DNA with *Msp* I generated a number of poorly resolved low molecular weight bands (Fig. 2). *Hpa* II digestion yielded the same pattern of low molecular weight bands and a number of high molecular weight bands, indicating that the TK genes in line 101 were differentially methylated. Some gene copies contained little or no methylation, whereas others were more heavily methylated.

Digestion of 101BU1 DNA with *Msp* I yielded the same pattern of low molecular weight bands as seen in 101 DNA, whereas *Hpa* II digestion generated only high molecular weight bands. The low molecular weight bands indicative of unmethylated TK genes were not visualized. These data indicate that sites within the plasmid sequences that were not methylated in 101 had become methylated in the DNA of the TK-deficient line 101BU1. The low molecular weight bands reappeared in *Hpa* II digests of DNA isolated from the reexpressing cell lines 101H1, 101HC, and 101HG, indicating that at least one copy of the TK gene in the DNA of these lines had been demethylated.

Sma I, another methylation-sensitive enzyme, recognized five sites within plasmid pTKx-1 to generate bands of 0.2, 0.25, 0.8, 1.6, and 5.0 kb (Fig. 3) (M. Wagner and W. Summers, personal communication). The 0.8- and 1.6-kb bands are derived entirely of TK DNA, and the sites that generate them lie within and on both sides of the structural gene. The 5.0-kb band consists of pBR322 and a small amount of TK DNA. The 0.8-, 1.6-, and 5.0-kb bands can be seen clearly in DNA of line 101 and the reexpressors 101H1, 101HC, and 101HG after *Sma* I digestion (Fig. 3) but are not detectable in 101BU1 DNA. Digestion with *Ava* I, another methylation-sensitive enzyme that recognizes eight sites in pTKx-1 (M. Wagner and W. Summers, personal communication), also demonstrated that the TK DNA of 101BU1 is more heavily methylated than is the TK DNA in 101 and in the TK^+ reexpressors. Two bands of 0.64 and 0.78 kb, derived from within the TK gene, were present in 101 and

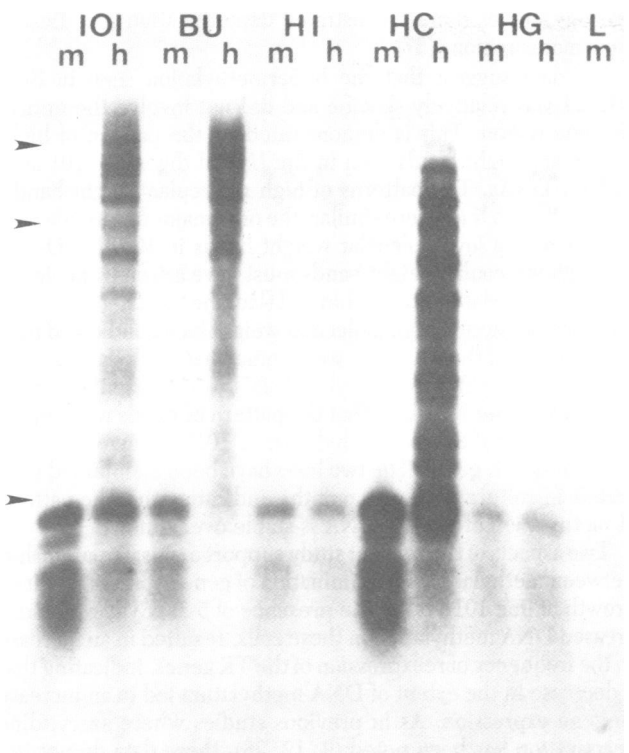

FIG. 2. *Msp* I and *Hpa* II digests of line 101 and derivatives. DNA of lines 101, 101BU1, 101H1, 101HC, and 101HG were digested with *Msp* I (lanes m) and *Hpa* II (lanes h). LTK$^-$ (lane L) DNA was digested with *Msp* I. DNA (10 μg) from each cell line was electrophoresed on a 1.2% agarose gel, transferred to nitrocellulose, and hybridized with ^{32}P-labeled pTKx-1 as a probe. Some of the TK sequences in 101 are methylated, as shown by the many high molecular weight bands in the *Hpa* II digest. Low molecular weight bands similar to those seen in the *Msp* I digest are also present, demonstrating the presence of unmethylated *Hpa* II sites within one or more TK genes. The TK sequences of 101BU1 are heavily methylated, and no low molecular weight bands appear in the *Hpa* II digest. The low molecular weight bands are present in both *Msp* I and *Hpa* II digests of 101H1 and 101HG, indicating that at least one copy of the TK gene was demethylated. In 101HC, the presence of high molecular weight bands in the *Hpa* II digest indicates that some methylation is present, but the low molecular weight bands seen in the *Msp* I digest are also present, indicating that the TK DNA in 101HC is less methylated than that in 101BU1. Arrows indicate marker bands of 9.7, 4.3, and 0.5 kb.

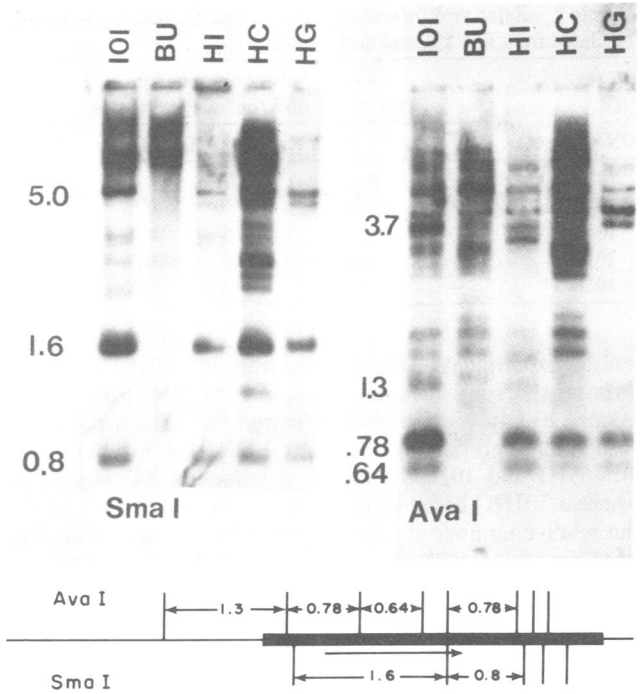

FIG. 3. *Sma* I and *Ava* I digests of line 101 and derivatives. DNA of lines 101, 101BU1, 101H1, 101HC, 101HG, and LTK$^-$ were digested with the enzymes *Sma* I or *Ava* I, both of which are sensitive to methylation at the CpG sequence. The DNAs were separated on 1.2% agarose gels, transferred to nitrocellulose, and hybridized with ^{32}P-labeled 3.5-kb HSV TK fragment as a probe. A partial restriction map showing the *Sma* I and *Ava* I cleavage sites within pTKx-1 is shown at the bottom of the figure. The 3.5-kb HSV TK fragment cloned into the *Bam*HI site of pBR322 is depicted as a heavy line. The location and direction of transcription of TK mRNA are represented by the arrow. The 5.0-, 1.6-, and 0.8-kb bands in the *Sma* I digests result from cleavage of pTKx-1 within and immediately flanking the TK gene. The bands are absent from 101BU1 DNA but are present in all DNA from cells expressing TK, indicating that the sites that generated these bands must have been methylated in the DNA of 101BU1 but unmethylated in the DNA of 101 and the reexpressing lines. Similarly the 0.64-, 0.78-, and 1.3-kb bands in the *Ava* I digest, derived from cleavage within the HSV TK fragment, are absent in 101BU1 DNA but present in DNA of all of the TK$^+$ lines.

in the reexpressors but absent from 101BU1 (Fig. 3). These data demonstrate that at least one copy of the TK gene within the DNA of each of the four TK$^+$ lines contained unmethylated *Hpa* II, *Sma* I, and *Ava* I sites and that those sites were methylated in the DNA of 101BU1.

Frequency of Reexpression of TK in Line 101BU1 Was Increased by Growth in the Presence of 5-azaCyd. 5-azaCyd is a cytidine analog that is incorporated into DNA and inhibits methylation. Treatment of 101 cells with 5-azaCyd decreased the extent of DNA methylation as judged by ethidium bromide staining of *Msp* I- and *Hpa* II-digested DNA after electrophoresis through 1% agarose gels (data not shown). Growth of 101BU1 cells in 2, 5, or 10 μM 5-azaCyd for 3–5 days prior to selection in HAT medium resulted in a 6- to 23-fold increase in the number of TK$^+$ colonies relative to untreated controls (Table 1), which suggests that DNA methylation is causally related to the elimination of gene expression in these cells. Maximal increase was seen after treatment with 5 μM 5-azaCyd for 3–4 days. The TK genes in line 101HC, which was derived from 101BU1 after 5-azaCyd treatment, were hypomethylated relative to the TK genes in 101BU1.

DNA of Line 101BU1 Was Unable To Transfer TK Activity in Secondary Gene Transfer. If DNA methylation directly prevents expression of the TK genes in line 101BU1, then DNA isolated from 101BU1 should not be active in secondary gene

Table 1. Increase in TK reexpression after treatment with 5-azaCyd

5-azaCyd treatment		Ratio positive/total plates		Colonies per 10^6 cells		
Dose	Time	No drug*	With drug*	No drug*	With drug*	Fold increase
5 μM	3 day	3/5	8/10	0.59	6.4	10.88
5 μM	3 day	4/10	10/10	0.27	2.7	10.0
5 μM	4 day	3/5	9/10	0.27	6.33	23.35
5 μM	5 day	9/10	10/10	0.40	5.68	14.2
5 μM	5 day	4/10	10/10	0.27	3.3	12.2
2 μM	3 day	4/10	10/10	0.27	1.6	5.9
10 μM	4 day	9/10	10/10	0.40	2.9	6.6

* 5-azaCyd treatment.

Table 2. Relative efficiencies of transfer of TK activity of DNA isolated from line 101 and derivatives

DNA source	Gene transfer experiments, no. of colonies/no. of Petri plates				
	1	2	3	4	Total
101	105/5	191/5	1/5	58/5	355/20
101BU1	0/5	0/5	0/10	0/5	0/25
101H1	—	12/5	72/5	14/5	98/15
101HC	—	—	161/5	70/5	231/10
101HG	—	—	17/5	9/5	26/10

transfer experiments. DNA isolated from 101, 101BU1, and the three reexpressors was applied to Ltk$^-$ cells. DNA from 101 and from each of the reexpressors generated TK$^+$ colonies, whereas each of three preparations of 101BU1 DNA was inactive (Table 2). 101H1 and 101HG suffered deletions of TK sequences, whereas 101HC had a gene amplification (see below), so that the relative number of colonies generated by each of the TK$^+$ lines correlated with the relative number of gene copies as judged from autoradiographs (Figs. 1–3).

DNA Rearrangements Accompanied Demethylation and Gene Reexpression. Each of the TK$^+$ reexpressors had alterations in the pattern or intensity of TK-specific bands after digestion with *Pvu* II (not shown), *Xba* I, *Hin*dIII, and *Bam*HI (Fig. 1). DNA of line 101H1 contained an additional band of about 10 kb after *Xba* I digestion and two additional bands after *Hin*dIII digestion. Additionally, bands at 17 and 5 kb were reduced in intensity relative to other bands after *Hin*dIII digestion, and at least two high molecular weight bands were undetectable after *Bam*HI digestion. DNA of line 101HC had a pattern of bands indistinguishable from that of line 101 except that the intensity of the bands, indicative of the number of gene copies, was increased. DNA from line 101HG was missing all but the 7.9-kb and 3.5-kb *Hin*dIII bands and had alterations in the high molecular weight region after *Bam*HI digestion. In order to determine if the alterations in gene structure were correlated with changes in gene expression, 12 subclones of 101BU1 were isolated randomly and without selection for TK expression. DNA from nine of the subclones was indistinguishable from 101BU1 DNA after *Hin*dIII digestion, whereas DNA from three of the subclones had changes in the pattern of TK-specific bands.

DISCUSSION

We have characterized a cell line, 101, which contains and expresses the HSV TK gene. Selection of 101 cells against expression of TK resulted in the appearance of derivatives that retained but did not express the TK genes. Derivatives of these subsequently were selected that reexpressed the TK gene.

In line 101 and its derivatives, expression of the TK gene is regulated by methylation. The TK genes in 101 are partially methylated, but as seen from the *Hpa* II, *Sma* I, and *Ava* I digests, at least one TK gene contains sites for these enzymes that are unmethylated. In line 101BU1, which does not express the TK genes, some of the *Hpa* II, *Sma* I, and *Ava* I sites that were unmethylated in 101 have become methylated, and those same sites have become demethylated in derivatives of 101BU1 that reexpress the genes, thus demonstrating an inverse correlation between DNA methylation and expression of the TK genes. The *Sma* I and *Ava* I digestions demonstrated that sites on the 5′ and 3′ ends of the TK gene, as well as within structural gene sequences, were unmethylated in the TK$^+$ lines and methylated in 101BU1, indicating that the pattern of methylation throughout the gene was altered. These results are in contrast to some previous studies that demonstrated demethylation specifically of promoter regions (27).

Our data suggest that the hypermethylation seen in line 101BU1 was relatively specific and did not involve the entire TK gene region. This is demonstrated by the pattern of high molecular weight bands seen in the *Hpa* II digests of 101 and 101BU1 DNAs. The patterns of high molecular weight bands seen in the two lines were similar; the only major difference was the absence of low molecular weight bands in 101BU1 DNA. The high molecular weight bands must have arisen from cleavage at unmethylated sites within and flanking the TK genes. The presence of discrete high molecular weight bands indicated that at least some of the TK genes were somewhat methylated, even in line 101, and that unmethylated sites were present, even in the DNA of line 101BU1. That the pattern of bands was similar indicated that the hypermethylation in 101BU1 involved only some of the TK genes. The two lines have been maintained separately in culture for over 9 months, indicating that the pattern of methylation of the TK DNA is stable over time.

Two aspects of the present study support a causal relationship between methylation and elimination of gene expression. First, growth of line 101BU1 in the presence of 5-azaCyd, which decreased DNA methylation in these cells, resulted in an increase in the frequency of reexpression of the TK genes, indicating that a decrease in the extent of DNA methylation led to an increase in gene expression. As in previous studies where azacytidine reactivation has been noted (9, 12, 28), these data do not unambiguously determine whether demethylation of the gene and flanking sequences, demethylation of other sequences, or even mutational events induced by 5-azaCyd are the important events in gene reexpression. For this reason, DNA isolated from 101, 101BU1, and from each of the three reexpressors was used in secondary gene transfer. The TK genes in 101BU1 DNA were inactive, whereas those in 101 DNA were active. Because gene transfer separates the TK DNA from most of the donor genome and from other cellular constituents (29), these data suggest that the difference lies within or near the TK genes themselves. The only detectable difference between the TK genes in lines 101 and 101BU1 is the extent of DNA methylation.

Each of three TK$^+$ derivatives of line 101BU1 had alterations in the structure of the TK genes. In two cases a deletion was evident, whereas in a third case a gene amplification was detected. DNA from 9 of 12 subclones of 101BU1 isolated without selection had band patterns indistinguishable from 101BU1 after *Hin*dIII digestion, whereas the pattern in 3 subclones differed. The presence of detectable rearrangements in the DNA of 3 of 12 random subclones indicates that rearrangement in the region of the introduced TK genes is a common event. We have not determined yet whether the gene rearrangements seen in the reexpressors provide a means of demethylation or are separate events. It may be that some rearrangements bring the methylated TK genes into proximity with hypomethylated DNA and, therefore, that the pattern of methylation of the TK genes is disrupted. Alternatively, demethylation may occur without gene rearrangement but is not in itself sufficient for gene reexpression, so that subsequent rearrangements are necessary for expression. Finally, DNA rearrangement may occur randomly and without relation to gene reexpression.

Gene transfer has been used previously to study the relationship between DNA methylation and gene expression (30–32). In one study in which the chicken TK gene was methylated *in vitro* and used in gene transfer experiments, the frequency of transfer was 5–33% of that with the unmethylated gene. The authors estimated that the pattern of methylation of transferred genes was lost at about 9% per generation (32). A second publication indicated that there was an initial demeth-

ylation of transferred genes, but the pattern subsequently was maintained faithfully (30). Our data indicate that the frequency of transfer of methylated genes was reduced to less than 1/200th that of the hypomethylated genes, and further, that the pattern of methylation of the transferred genes was altered sufficiently to alter gene expression only at a frequency of 5×10^{-7}. We feel that the differences between our results and the previous studies stem from the fact that genes in the first studies were methylated *in vitro* only at *Hpa* II sites, which represent, on the average, only 6% of all potential methylation sites and may or may not be sites that are important for gene control. In the experiments reported here, the methylation was carried out by the normal cellular machinery *in vivo* so that the extent of methylation was likely to be greater, and the pattern of methylation was likely to be that which abolished gene expression.

Although the DNA of line 101BU1 was unable to generate TK^+ colonies in a number of independent gene transfer experiments, DNA from line 101 and from each of the three reexpressors could generate TK^+ colonies. The frequency of colonies generated by DNA of lines 101H1 and 101HG was reduced relative to that obtained with 101 and 101HC, suggesting that they had fewer active gene copies. This interpretation is consistent with the intensity and diversity of bands visualized on Southern blots.

Thus, our data demonstrate that changes in the pattern of methylation that effected changes in gene expression occurred throughout the TK gene. The secondary gene transfer experiments demonstrated that methylation of the structural gene and of flanking DNA was sufficient to abolish expression. However, our data do not permit a determination of whether there are particular sites of methylation within a gene that are important for controlling gene expression or whether methylation of the entire gene, perhaps altering the conformation of the DNA, is the important factor.

We thank K. M. Huttner for communication of unpublished results; Dr. E. Moudrianakis, Dr. R. C. Huang, and G. Ketner for helpful discussions; and Diane Warr for typing of the manuscript. This work was supported by a biomedical research grant from the National Institutes of Health. This is contribution no. 1173 from the Department of Biology.

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