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Phenotypic Switching in Cells Transformed with the Herpes Simplex Virus Thymidine Kinase Gene

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Biochemical transformation of Ltk$^-$ cells with the herpes simplex virus thymidine kinase (*tk*) gene resulted in numerous TK$^+$ colonies that survived selection in hypoxanthine-aminopterin-thymidine medium. Many of these TK$^+$ cell lines switched phenotypes and reverted to the TK$^-$ state. In this report, we describe the biological and biochemical characteristics of three TK$^-$ revertant lines. One (K_1B_5) transiently expressed TK in the presence of bromodeoxyuridine, which selects for the TK$^-$ phenotype. Another TK$^-$ sibling ($K_1B_6^n$) expressed TK only after removal from bromodeoxyuridine-containing medium. The last variant ($K_1B_6^{me}$) lost the ability to switch to the TK$^+$ phenotype, although it maintained the herpes simplex virus sequences coding for TK. Loss of the ability of $K_1B_6^{me}$ cells to express TK was correlated with extensive methylation of the sequence recognized by the restriction endonuclease *Hpa*II (pCpCpGpG). After these cells were treated with 5-azacytidine, they regained the ability to clone in hypoxanthine-aminopterin-thymidine medium and reexpressed virus *tk* mRNA and enzyme. In addition, the *Hpa*II sites that were previously shown to be refractile to enzyme digestion were converted to a sensitive state, demonstrating that they were no longer methylated.

Biochemical transformation of mammalian cells by use of isolated DNA fragments generated by restriction endonuclease cleavage provides a unique system with which to study gene expression in higher eucaryotes. One example of this approach is the introduction of the herpes simplex virus *tk* gene into *tk*$^-$ mouse cells and selection of biochemically transformed cells in medium containing HAT (10, 24). A class of mutants exists that switches from the TK$^+$ to the TK$^-$ phenotype and is readily selected with the appropriate growth medium (19). These cell lines afford us the opportunity to examine the fine structure of the *tk* gene after such a phenotypic switch has occurred and to ask whether local changes at the molecular level can be correlated with switching. These mutants switched to the TK$^-$ phenotype when grown in BUdR-containing medium and were shown to switch back to the TK$^+$ phenotype at a relatively high frequency (1 to 50%) when selected in medium containing HAT. This situation is in contrast to that described by Davidson et al. (6). The transformants they studied were selected after infection with UV-inactivated herpes virus, and subsequently a class of TK$^-$ mutants was derived after growth in medium that contained BUdR. However, when these cells were grown in HAT medium, they reverted at low frequency to the TK$^+$ phenotype (10^{-6} mutants per cell per generation), although the *tk* gene is maintained in these lines (S. Silverstein and R. L. Davidson, unpublished data). These cells have recently been shown to have methylated the DNA sequences around the integrated *tk* gene (D. W. Clough and R. L. Davidson, submitted for publication).

One of the mutant cell lines of particular interest to us, which we have designated $K_1B_6^{me}$, resembles the class of mutants derived by Davidson et al. (6) rather than the high-frequency switchers that we have studied. This cell line, which was derived from a TK$^-$ revertant after continuous passage in BUdR, has lost the ability to reexpress the gene coding for the TK$^+$ phenotype, although it retains *tk* sequences (19). Furthermore, DNA derived from the $K_1B_6^{me}$ cell line is unable to serve as a donor of TK activity in the transformation of *tk*$^-$ cells.

The restriction enzymes *Hpa*II and *Msp*I recognize and cleave the sequence pCpCpGpG. However, when the cytosine in the pCpG sequence is methylated, cleavage by *Hpa*II but not by *Msp*I is inhibited (2, 3, 8, 12). Digestion of DNA from primary *tk*$^+$ transformants with either of these restriction endonucleases results in identical hybridization profiles in Southern blots with the characteristic low-molecular-weight DNA fragments of the *tk* gene. Digestion of DNA from $K_1B_6^{me}$ cells with *Msp*I results in this

characteristic restriction pattern. However, after digestion with *Hpa*II, no small DNA fragments are detected; rather, they are replaced by larger ones, suggesting that in this cell line, methylation of *Hpa*II sites within the *tk* gene has occurred (19).

We sought to test the hypothesis that the methylation of eucaryotic DNA, specifically at cytosine residues, is involved in the regulation of gene expression by culturing $K_1B_6^{me}$ cells in the presence of 5-azaC, a drug which purportedly causes the hypomethylation of DNA by substituting for cytosine residues that can be methylated (9). Such treatment should allow the reexpression of the dormant *tk* gene in these cells if methylation of DNA at cytosine residues within or about the gene is a means of regulating the expression of TK. The experiments described here demonstrate that after exposure to 5-azaC, these phenotypically TK^- cells can regain the ability to express TK and that this probably results from hypomethylation of *tk* DNA sequences rather than from the mutagenic effect of 5-azaC. In addition, we compared the ability of this cell line ($K_1B_6^{me}$) and its 5-azaC-induced revertants with cells that readily switch TK phenotypes and showed that "switchers" can be differentiated into two groups, those which constitutively express TK and those which express TK only when grown in the absence of BUdR.

MATERIALS AND METHODS

Abbreviations. We have used the abbreviation TK to refer to thymidine kinase protein, TK^+ or TK^- to indicate the phenotype of cell lines, and *tk* to indicate genotype or when referring to nucleic acid (DNA or mRNA) coding for thymidine kinase. Other abbreviations are: HAT, hypoxanthine (15 μg/ml), aminopterin (1 μg/ml), and thymidine (5 μg/ml); HT, hypoxanthine (15 μg/ml) and thymidine (5 μg/ml); BUdR, bromodeoxyuridine; 1× SSC, 150 mM NaCl plus 15 mM sodium citrate; [^{3}H]TdR, [^{3}H]thymidine; 5-azaC, 5-azacytidine.

Growth of cells. Cells were grown in Dulbecco modified eagle medium supplemented with 10% calf serum. BUdR (30 μg/ml), HAT, or HT was added to some of the cultures as required.

Cell pedigree. Ltk^- cells were transformed to the TK^+ phenotype with a unique 5.0-kilobase *Kpn*I fragment from herpes simplex virus type 1 strain F DNA. A clone selected in HAT medium was isolated and designated LHK_1. This clone was subcloned and selected in BUdR-containing medium. Seven individual BUdR-resistant revertant clones were isolated and expanded in BUdR. Their restriction endonuclease profiles were determined, and two were chosen for further study. K_1B_5 was shown to have undergone rearrangement of the *tk* sequences, whereas K_1B_6 maintained the blot hybridization pattern of its parent K_1 (19). Each of the revertant clones was frozen in liquid nitrogen shortly after isolation. Stocks of each clone were maintained in the laboratory in the presence of selective pressure (BUdR) for almost 2 years before their analysis. K_1B_5 cells had maintained their ability to switch to the TK^+ phenotype, whereas K_1B_6 cells had lost the ability to switch. These cells were designated $K_1B_6^{me}$. The parent cell line frozen shortly after isolation maintained the ability to switch and was designated $K_1B_6^n$.

Isolation and size analysis of *tk* RNA. Cells were grown in roller bottles in medium as indicated below until confluent (3×10^8 cells per bottle), scraped into sterile phosphate-buffered saline and washed two times with phosphate-buffered saline. Cell pellets were suspended in buffer containing 20 mM Tris-hydrochloride (pH 7.5), 2 mM $MgCl_2$, and 3 mM $CaCl_2$ (5 ml per roller bottle). Nonidet P-40 was added to a final concentration of 0.5% and the cell suspension was gently homogenized with a Dounce homogenizer. Nuclei were pelleted by centrifugation, the supernatant containing cytoplasmic RNA was removed, and sodium dodecyl sulfate was added to 0.1%. The supernatant was then extracted two times with phenol and two times with chloroform-isoamyl alcohol (24:1, vol/vol), and the RNA was precipitated at −20°C after the addition of NaCl to 0.4 M and 2 volumes of ice-cold ethanol. Infected-cell RNA was prepared in this manner, using herpes simplex virus type 1 (strain F)-infected CV-1 cells. Confluent roller bottles of CV-1 cells were overlaid with 15 ml of Dulbecco medium containing 1% heat-inactivated calf serum and virus (20 to 30 PFU per cell) for 1.5 h. At this time, the medium containing virus was removed and replaced with 50 ml of fresh Dulbecco medium containing 1% heat-inactivated calf serum for a period of 6 to 8 h, after which the cells were harvested. Polyadenylated cytoplasmic RNA was selected by chromatography on oligodeoxythymidylate-cellulose columns. Alternatively, whole-cell RNA was extracted after cell pellets were suspended in 4 M guanidine thiocyanate–0.5% *N*-lauryl sarcosine–0.1 M β-mercaptoethanol by the method of Chirgwin et al. (4).

RNA was electrophoresed in 0.8% agarose gels containing 2.2 M formaldehyde, and 1 μg of ethidium bromide per ml was added to permit visualization of rRNA size markers. Electrophoresis buffer contained 20 mM morpholinoethane sulfonic acid, 5 mM sodium acetate, and 1 mM EDTA (pH 7.0). Before sample loading, RNA was incubated to 60°C for 5 min with 50% formamide and 2.2 M formaldehyde. RNA was transferred to nitrocellulose filters, essentially as described by Southern with 10× SSC and then was baked for 5 h in vacuo at 80°C. Nitrocellulose filters were prehybridized for 12 h at 45°C in plastic bags with 50% formamide, 10% dextran sulfate, 6× SSC, 0.1% sodium dodecyl sulfate, 0.02% Ficoll, 0.02% bovine serum albumin, and 0.02% polyvinylpyrrolidone. Filters were hybridized in a solution containing 50% formamide, 10% dextran sulfate, 2× SSC, 10 mM EDTA, 5 μg of salmon sperm DNA per ml, 0.1% sodium dodecyl sulfate, and 10 to 15 ng of ^{32}P-labeled *tk* DNA probe (3×10^8 cpm/μg) at 45°C for 12 h. The filters were then washed as described below except that the first wash in 2× SSC was done at room temperature for 30 min.

Autoradiographic assay for *tk* activity. Cells were plated at low density on microscope slides and grown for 5 to 10 generations in growth medium. The cells were washed extensively with phosphate-buffered sa-

line, and the medium was replaced with Dulbecco medium containing 5 μCi of [^{3}H]TdR per ml for 3 to 4 h. The microscope slides were washed extensively with phosphate-buffered saline, fixed for 20 min with methanol-acetic acid (3:1, vol/vol), dipped in Kodak NTB nuclear track photographic emulsion, and stored in a light-tight box for 48 h at 4°C. The photographic emulsion on the slides was developed for 2.5 min in Kodak liquid X-ray developer and fixed for 5 min in Kodak Rapid Fixer. The cells were then counterstained with Giemsa stain for 5 min to permit visualization of unlabeled cells. Cells were counted and photographed at a magnification of 400× using an Olympus microscope and an OM-2 camera with Kodak Plus-X film.

Filter hybridization. DNA was extracted from transformed cells and aza-C revertants as previously decribed (17). The DNAs were digested with either *Hpa*II or *Msp*I and electrophoresed on 1.4% agarose gels. The DNA was depurinated by exposure to 0.2 M HCl for 20 min, denatured with 0.5 M NaOH, and then soaked in Tris-buffered 20× SSC for 1 h before transfer to nitrocellulose paper. The filters were baked for 4 h at 80°C in vacuo, blocked in 1× Denhardt solution (0.02% each of Ficoll, polyvinylpyrrolidone, and bovine serum albumin in 6× SSC), and hybridized to ^{32}P-labeled *tk* DNA at 68°C for 14 h. The filters were washed successively in 2× and 0.2× SSC twice for 15 min each at 68°C. The dried filters were exposed to Dupont Cronex 2DC film at −80°C with a single intensifying screen.

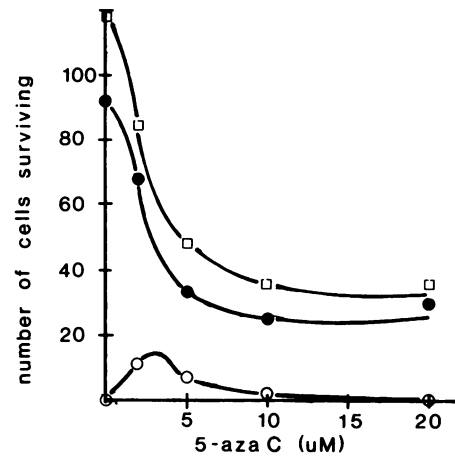

FIG. 1. Toxicity of 5-azaC on transformed cells. Cells were grown in various concentrations of 5-azaC for 48 h and then in drug-free medium for 48 h. The cells were then replated (100 cells per plate), and the number of surviving colonies as a function of drug concentration was determined. Each point on the graph represents an average of three plates of cells. Symbols: □, Dulbecco medium; ●, BUdR; ○, HAT (10^5 cells per plate).

RESULTS

Effect of 5-azaC on transformed cells. We have previously demonstrated that the *Hpa*II sites within and about the *tk* gene in $K_1B_6^{me}$ cells are heavily methylated (19). In this experiment, we sought to determine whether the *tk* gene could be reactivated after exposure of these cells to 5-azaC. We examined the toxic effects of 5-azaC on $K_1B_6^{me}$ cells after growth for 48 h in medium containing various concentrations of 5-azaC. After 48 h, the medium was replaced, and the cells were allowed to grow in the absence of the drug for 48 h to permit replication of substituted DNA. Cells were then replated in either nonselective medium or medium selecting for (HAT) or against (BUdR) TK expression, and the number of surviving colonies was determined (Fig. 1). When cells were plated in either nonselective or BUdR-containing medium, progressively fewer surviving colonies were detected as a function of increasing 5-azaC concentration. The maximal number of HAT-resistant colonies appeared after growth in 10 μM 5-azaC. Higher concentrations of drug resulted in no further elevation in the proportion of HAT-resistant colonies.

Although exposure of $K_1B_6^{me}$ cells to 5-azaC gave rise to the appearance of colonies which survived in HAT medium, the frequency of stable TK^+ revertants was low (10^{-5}), perhaps reflecting the necessity of converting all or many of the large number of methylated sites within and about the *tk* gene.

Change in methylation pattern in 5-azaC-induced revertants. We have used the isoschizomers *Hpa*II and *Msp*I to distinguish DNA sequences which contain methylcytosine in the sequence pCpCpGpG from those that are not methylated at this site. To determine whether HAT-resistant clones that arose after growth in 5-azaC retained methylated residues at the *tk* locus, we digested DNA from $K_1B_6^{me}$ and two 5-azaC-induced revertants with either *Hpa*II or *Msp*I. The DNAs were then analyzed by blot hybridization. The annealing profile of each of the HAT-resistant revertants revealed the characteristic low-molecular-weight DNA fragments when the revertants were digested with either *Hpa*II or *Msp*I (Fig. 2). DNA from the nonexpressing cell line did not reveal any of the low-molecular-weight DNA fragments expected after *Hpa*II digestion. Instead, larger fragments were generated. The *Msp*I digestion pattern, on the other hand, was indistinguishable from that obtained after digestion of the cloned *tk* gene or the 5-azaC-induced cells with *Hpa*II.

RNA synthesis during phenotypic switching. A series of biochemical transformants grown in medium containing HAT, BUdR, or no additions was examined for the presence of *tk* mRNA. Cytoplasmic polyadenylated RNA was isolated from herpes simplex virus type 1-infected or transformed cells and analyzed by North-

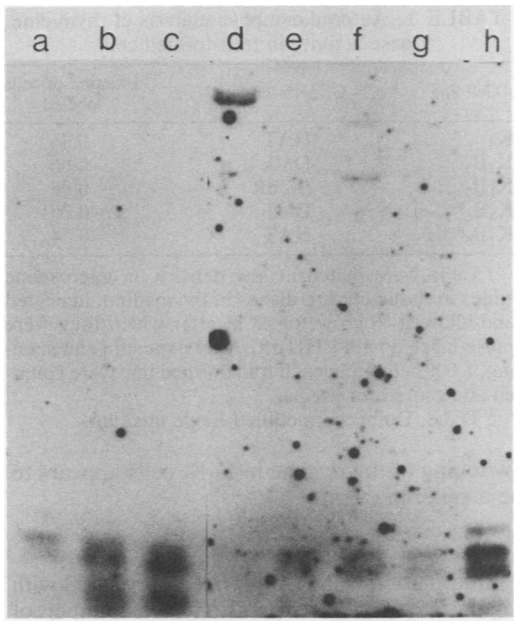

FIG. 2. Blot hybridization profile of *tk* DNA from transformed cells. DNA was isolated from a nonreverting tk^- cell and two HAT-resistant revertants isolated after growth in 5-azaC. The DNA (10 μg) was cleaved with either *Hpa*II (a,b,d,f) or *Msp*I (c,e,g,h) and electrophoresed on a 1.4% agarose gel, transferred to nitrocellulose paper, and hybridized to a ^{32}P-labeled *tk* DNA probe. (Lanes a and h) $_{p}tk$-5; (lanes b and c) K_1B_6, aza-1; (lanes f and g) K_1B_6aza-C; (lanes d and e) $K_1B_6^{me}$.

ern blot hybridization, using a cloned ^{32}P-labeled 875-base pair *Pst*I fragment contained entirely within the 5′ end of the *tk* mRNA (14, 22). Polyadenylated RNA extracted from cells was analyzed by formaldehyde agarose gel electrophoresis and blot hybridization as described above.

Figure 3 shows the size of *tk* RNA present in a number of *tk*-transformed cell lines. The hybridization pattern of transformed-cell RNAs expressed in these cell lines is shown in Fig. 3A, lane a. RNA obtained from a primary transformant (K_1), selected and maintained continuously in HAT-containing medium, expressed the 1.4-kilobase *tk* mRNA as well as a smaller RNA species of 900 nucleotides that is not normally detected in infected-cell RNA. One cell line ($K_1B_6^n$) exhibited the RNA switching pattern that would be expected from cloning efficiency experiments (19). These cells did not accumulate detectable amounts of *tk* mRNA when grown in medium containing BUdR. However, they regained the ability to express *tk* mRNA when grown in neutral medium (Fig. 3A, lanes b and c). $K_1B_6^n$ cells apparently controlled TK switching at the level of transcription.

K_1B_5 cells also synthesized *tk* mRNA when grown in neutral medium. However, contrary to our expectations, this cell line expressed *tk* mRNA when cultured in medium containing BUdR (Fig. 3A, lane e). Two possible explanations for this anomaly occur to us: either the mechanism responsible for the phenotypic switch is unstable, allowing a population of TK-expressing cells to arise spontaneously in the presence of BUdR, or the K_1B_5 cell line regulates TK expression posttranscriptionally at the level of translation. RNA extracted from the nonreverting cell line $K_1B_6^{me}$ is shown in Fig. 3A, lane d; these cells did not accumulate detectable amounts of *tk* mRNA in neutral medium.

Analysis of RNA from HAT-resistant clones derived after exposure of $K_1B_6^{me}$ to 5-azaC reveals the presence of a species of *tk* mRNA that is indistinguishable in size from authentic *tk* mRNA (Fig. 3B, lane d). Thus, these cells regained the capacity to transcribe authentic *tk* mRNA.

TK activity in cells undergoing phenotypic switching. Levels of TK enzyme in transformed cells are often too low to be detected by standard enzyme assays. Therefore, we qualitatively analyzed enzyme activity by autoradiography of cells that were pulse-labeled with [^{3}H]TdR; in this way, extremely low levels of TK activity could be detected. Cells grown on microscope

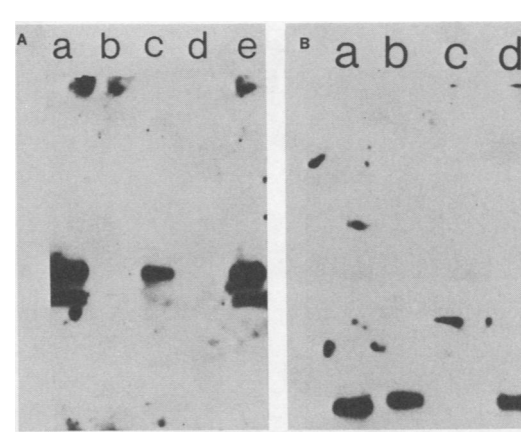

FIG. 3. Annealing profile of *tk* mRNA from transformed cells. Polyadenylated RNA (5.0 μg) isolated from transformed cells was electrophoresed through formaldehyde gels and analyzed for the presence of *tk* sequences by blot hybridization with a ^{32}P-labeled DNA probe contained entirely within the coding region of *tk*. (A) RNA from K_1 cells (a), RNA from $K_1B_6^n$ cells propagated in BUdR (b) or neutral medium (c), RNA from $K_1B_6^{me}$ (d), RNA from K_1B_5 cells grown in BUdR (e). Figure (B) RNA (0.5 μg) from herpes simplex virus type 1-infected cells (a), RNA from K_1, $K_1B_6^{me}$, and an aza-C-induced revertant $K_1B_6^{me}H_2$, lanes b, c, and d, respectively.

slides in medium containing HT ($K_1B_6^{me}$) or HAT ($K_1B_6^{me}$aza-C) were pulse-labeled with [^{3}H]TdR for 3 h and analyzed by autoradiography for their ability to incorporate label into cellular DNA. The results of this experiment showed that 65% of the $K_1B_6^{me}$aza-C cell population incorporated label, whereas <0.1% of the $K_1B_6^{me}$ cell population incorporated label (Fig. 4B and C). Longer pulses (12 h) were employed to increase the sensitivity of the assay. Over 90% of $K_1B_6^{me}$aza-C cells had incorporated [^{3}H]TdR, but no $K_1B_6^{me}$ cells had grains (Table 1).

TK activity was also assayed in K_1B_5 cells maintained in medium containing BUdR. These cells incorporated [^{3}H]TdR into their DNA (Fig. 4D). Thus, they made active TK protein, confirming our observation that these cells synthesize *tk* mRNA when grown in medium containing BUdR. This experiment enables us to argue strongly that posttranscriptional regulation is not the mechanism controlling phenotypic switching in this cell line. We also plated these cells at very low density and allowed them to grow into discrete colonies which were pulse-labeled with [^{3}H]TdR. We observed that the vast majority (>75%) of clones contained [^{3}H]TdR-labeled cells (data not shown). Thus, the on-off switching of the *tk* gene in K_1B_5 cells appears to be a random event.

TABLE 1. Autoradiographic analysis of thymidine kinase activity in transformed cells[a]

Cell line	Growth medium	Fraction of cells labeled
K_1	HAT	0.46
K_1B_5	DME[b]	0.06
K_1B_5	BUdR	0.06
$K_1B_6^{me}$	DME	<0.001
$K_1B_6^{me}H_2$	HAT	0.54

[a] Cells were plated at low density on microscope slides in tissue culture dishes in the medium indicated and allowed to grow for 48 h, after which they were pulse-labeled with [^{3}H]TdR. After exposure and staining, 1,000 cells from each transformed line were counted and scored for grains.

[b] DME, Dulbecco modified Eagle medium.

DISCUSSION

Methylation of DNA has been correlated with regulation of gene expression in a number of systems. For example, lymphoid cells immortalized by *Herpesvirus saimiri* contain multiple copies of viral DNA, a portion of which are episomal. The viral DNA sequences in the cell lines 1670 and 70N2, which do not produce

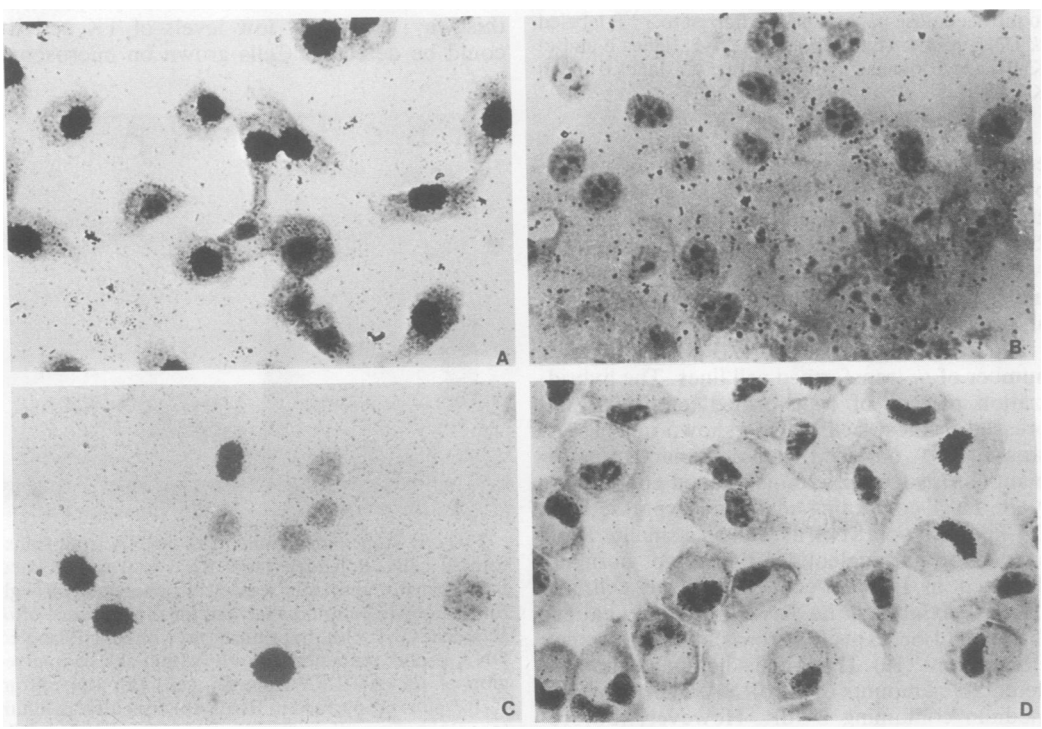

FIG. 4. TK activity in transformed cells. Cells were seeded onto microscope slides and allowed to grow for 5 to 10 generations. They were then pulse-labeled with 5 μCi/ml of [^{3}H]TdR, fixed, and exposed to NTB-2 emulsion. (Panel A) K_1 maintained in HAT; (panel B) $K_1B_6^{me}$ grown in neutral medium; (panel C) $K_1B_6^{me}H_2$ in HAT; (panel D) K_1B_5 grown in BUdR.

detectable virus, are extensively methylated, whereas the viral sequences from three virus-producing cell lines are not (7). An analogous finding in adenovirus type 12-transformed hamster cells has been described by Sutter and Doerfler (18). The integrated viral sequences in four adenovirus type 12-transformed hamster cell lines were shown to be extensively methylated. Early virus genes, which are expressed as mRNA, were found to be hypomethylated when compared with late virus gene sequences, which were heavily methylated and not expressed. Weintraub et al. (23) have shown that methylation patterns of DNA in the chicken α-globin gene cluster are altered during development. Active genes are not methylated and are DNase I-hypersensitive, whereas 5′ and 3′ flanking sequences are methylated and insensitive to DNase I. Inactivation of an embryonic α-globin gene during development was associated with the methylation of coding sequences and loss of DNase I hypersensitivity. A number of other systems have been described which associate the methylation of DNA with gene inactivation, such as β-globin (13, 21), ovalbumin (11), ribosomal genes (1, 15), and the metallothionein I gene in mice (5).

The drug 5-azaC has been used to reactivate quiescent genes by a mechanism that results in the hypomethylation of DNA sequences. Treatment of human-mouse hybrid cells with 5-azaC results in the expression of the X-linked hypoxanthine-guanine phosphoribosyl transferase by reactivation of the X chromosome (16). Taylor and Jones (20) have demonstrated that cultured cells express differentiated phenotypes when grown in the presence of 5-azaC. Later, they correlated the induction of myogenesis in 10T1/2 cells with hypomethylation of *Hpa*II sites after drug treatment (9).

Selection of the TK^- phenotype from *tk*-transformed cells after growth in BUdR shows that these cells can switch phenotypes. When TK^- revertants are then selected for the TK^+ phenotype after growth in HAT, two patterns of switching are observed: cells either switch at high frequency or they switch very infrequently. Two cell lines which readily switch TK phenotypes have been studied in detail. $K_1B_6^n$ cells have a "simple" transcriptional switch which turns off *tk* mRNA synthesis when cells are grown in BUdR-containing medium; however, when these cells are cultured in medium containing HAT or HT, they synthesize *tk* mRNA. The transcriptional switch operant in K_1B_5 cells, however, appears to be unstable because of subpopulation of TK-expressing cells (around 10%) arises even when cells are grown in BUdR-containing medium. The cause of this unstable phenotype is not known, but it may be explained by the fact that the transforming viral DNA fragment has undergone some extensive sequence rearrangements (19).

The third mutant cell line that we examined does not normally revert to the TK^+ phenotype. Our results show clearly that extensive methylation of DNA sequences around the *tk* gene has occurred in this nonreverting cell line, $K_1B_6^{me}$. We cannot, at this point, determine whether this methylation event is the result of mutation or whether there is a functional correlation between methylation of specific *tk* sequences and loss of gene activity. The reactivation of the *tk* gene in these cells after treatment with 5-azaC and the concomitant loss of methylated *Hpa*II-*Msp*I restriction sites strongly support the hypothesis that the specific methylation and demethylation of DNA sequences can serve as a transcriptional control mechanism in eucaryotic cells.

We can rule out the possibility that the methylation of DNA in the $K_1B_6^{me}$ cell line is a general phenomenon peculiar to this line and is unrelated to gene regulation. An examination of methylatable sites in the β-globin gene in this cell line and the parental cell line (K_1) has shown that the *Hpa*II and *Msp*I digestion patterns are identical in the two cell lines (19). We have not yet correlated methylation and subsequent demethylation at specific *Hpa*II-*Msp*I restriction sites with transcriptional control. Our *Hpa*II-*Msp*I gels resolve only the largest fragments and not the many smaller fragments generated by cleavage with these enzymes. Some of these potential methylation sites lie upstream from the 5′ end of the *tk* gene and are regarded as the most likely sites where regulatory events might occur. The possibility also exists that the appearance of TK activity in 5-azaC-treated cells is a consequence of the mutagenic activity of the drug; for example, the drug may have effected the expression of dormant cellular TK. However, this is unlikely because the primary transformant from which the nonreverting cell line was generated expresses virus-specific TK, and the 5-azaC-treated cells also express viral *tk* mRNA.

In conclusion, we characterized three TK^- revertants that were isolated by BUdR selection from a common tk^+ progenitor (K_1). These TK^- revertants displayed three distinct phenotypes. $K_1B_6^n$ cells readily switched to the TK^+ phenotype when cultured in HAT medium. They accumulated *tk* mRNA only when grown in the absence of BUdR. The TK^- cell line K_1B_5 also switched at a high frequency. However, a subpopulation of these cells continued to synthesize *tk* mRNA and to express TK enzyme activity, even when maintained in BUdR. Autoradiographic studies of isolated clones of K_1B_5 cultured in BUdR showed that greater than 75% of

the clones contained labeled cells. Thus, the TK^+ cells in the K_1B_5 cell line were not derived from a mixed cell population. In addition, we predict that the prolonged period of time that these cells were maintained in BUdR medium would select against any TK^+ cells that constitutively express the *tk* gene. The TK^+ cells observed represented a random subpopulation that appeared to transiently express virus enzyme. These cells presumably were selected against during culturing in BUdR. In support of this hypothesis, we consistently observed that (i) colony morphology in BUdR was disperse, (ii) the number of cells accumulating after plating in nonselective medium was significantly (twofold) higher than when these cells were cultured in BUdR, and (iii) [^{3}H]TdR-labeled cells were observed in the majority of subclones generated from these cells. Neither $K_1B_6^n$ nor K_1B_5 showed any evidence for methylation of *tk*-specific sequences in its DNA (19).

The third revertant we examined, $K_1B_6^{me}$, had lost the ability to express TK. The *tk* sequences within this line were previously shown to be hypermethylated (19). Methylation of DNA did not appear to regulate transient on-off switching of genes, but rather seemed to be associated with a more lasting shutdown of gene activity, shunting some genes into a functionally inert state. Exposure of $K_1B_6^{me}$ cells to 5-azaC resulted in progeny that had regained the ability to express TK as judged by their capacity to grow in HAT-containing medium and to incorporate[^{3}H]TdR into cellular DNA. We have shown that the ability to reexpress TK activity is associated with hypomethylation of *Hpa*II sites in *tk* DNA and with the reappearance of functional viral *tk* mRNA.

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