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Genetics

Induction of prolactin-deficient variants of GH_3 rat pituitary tumor cells by ethyl methanesulfonate: Reversion by 5-azacytidine, a DNA methylation inhibitor

(alkylating agent/gene expression/peptide hormone/somatic cell genetics/differentiation)

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ABSTRACT **GH_3 cells are a rat pituitary tumor line expressing two pituitary peptide hormones, prolactin (rPRL) and growth hormone. Recently, it was found that the DNA alkylating agent ethyl methanesulfonate can induce the appearance of rPRL-deficient GH_3 cell variants at a high frequency (*ca.* 20–30%). As shown here, such variants cannot be induced at high frequency by irradiation of wild-type GH_3 cells with ultraviolet light, indicating that the effect may be specific to treatment with alkylating agents. Furthermore, the DNA methylation inhibitor 5-azacytidine reverted an ethyl methanesulfonate-induced rPRL-deficient variant into rPRL-expressing cells at high frequency (*ca.* 50%). The revertants were stable for at least 30–35 generations. These results support the hypothesis that the alkylating agent may promote the specific methylation of the rPRL gene or a gene regulating its activity, either one of which leads to inactivation of expression of the rPRL gene in GH_3 cells.**

GH_3 cells are an established tumor line isolated from a single estrogen-induced tumor of rat anterior pituitary (1, 2). They have been widely studied as a model system for hormone action on pituitary function because they express two pituitary polypeptide hormones (3), prolactin (rPRL) and growth hormone (rGH), both of which are under complex, multihormonal control (4–10). By producing both peptide hormones, however, GH_3 cells differ significantly from their normal pituitary cell counterparts which express one or the other polypeptide but not both (11).

Recently, we found (12) that treating GH_3 cells with the DNA alkylating agent ethyl methanesulfonate (EtMes) at a dose allowing 10–30% survival promoted the appearance of PRL-deficient variants at a high frequency (*ca.* 20–30%) with no effect on rGH expression. Both phenotypically stable and unstable variants were found. Biochemical analysis showed that, in the variants, the rate of rPRL synthesis had decreased by up to a factor of 1/100 and this was paralleled by a similar decline in the cytoplasmic levels of pre-rPRL mRNA. Because no rPRL-deficient clones were found in untreated cells, it was concluded that the alkylating agent was promoting a stable change in rPRL gene expression at high frequency through a nonmutational mechanism. One possibility was that EtMes increased the methylation of the rPRL gene or of an outside regulatory gene which subsequently blocked the expression of the rPRL gene. CpG sequences are relatively rare in mammalian DNA (13, 14) and are often methylated on the 5 position of cytosine in unexpressed genes (15–19). Importantly, patterns of 5-methylcytosine are heritable (20–22), and an effect of EtMes on DNA methylation was one way to explain the phenotypic stability of one of the variants.

We know of no reports describing an effect of EtMes on DNA methylation. Nonetheless, a major prediction of the model can be readily tested. If the alkylating agent promotes inactivation of the gene by DNA methylation, then its expression should be reactivated by agents that demethylate DNA. Here we report that a cytidine analogue, 5-azacytidine, that is incorporated into DNA and cannot be methylated because of a nitrogen at the 5 position of the pyrimidine (23), is able to revert an EtMes-induced rPRL-deficient variant to rPRL expression at high frequency. We also show that an alternative mutagenic treatment, UV irradiation, does not generate the deficient cells.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. The wild-type GH_3 line used in this study was cloned in 1976 from an original source (American Type Culture Collection); when initially isolated, it produced nearly equal levels of rPRL and rGH under standard culture conditions. B_3 cells are a rPRL-deficient variant of GH_3 cells cloned from the wild-type population after mutagenesis with EtMes (12). Both lines were grown routinely in monolayer cultures at 37°C in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal calf serum (GIBCO) in an incubator at 95% humidity under 5% CO_2 in air. Generation time under these conditions is about 41 hr.

Mutagenesis. Cells were mutagenized for 24 hr with EtMes (Sigma) at 300 μg/ml in the culture medium. Irradiation with UV light was performed with a GE germicidal lamp on monolayer cultures in Petri dishes from which the medium had been removed. Irradiation levels were monitored with a Blak-Ray UV meter (model J-225, Ultraviolet Products, San Gabriel, CA). Cells were grown for 4 days before they were cloned on the surface of 0.6% agar (Noble agar, Difco) in culture medium in bacterial Petri dishes as described (12). Individual wells of 24-well cluster dishes (Falcon or Costar) were used for growth to mass culture.

Assay of rPRL and rGH Production. Individual colonies were labeled with [^{35}S]methionine (usually for 3 hr) at 250 μCi/ml (1 Ci = 3.7×10^{10} becquerels) and samples of the medium were electrophoresed on 12.5% acrylamide/NaDodSO$_4$ slab gels as described (12). Labeled proteins were detected by autoradiography using Kodak XAR-5 x-ray film. To compensate for differences in cell density at the time of labeling, samples were loaded in proportion to the amount of the acid-insoluble radioactivity incorporated by each clone.

Immunoprecipitation. Samples of medium containing radioactively labeled rPRL and rGH were immunoprecipitated with

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Abbreviations: rPRL, rat prolactin; rGH, rat growth hormone (somatotropin); EtMes, ethyl methanesulfonate; HPRT, hypoxanthine phosphoribosyltransferase.

rhesus monkey anti-rGH antiserum (10) or rabbit anti-rPRL antiserum generously provided by the Rat Pituitary Hormone Distribution Program (National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases) and A. F. Parlow. Nonimmune rabbit or rhesus monkey sera were used for control precipitations, and immunocomplexes were isolated by indirect adsorption to SAC as described (24).

RESULTS AND DISCUSSION

5-Azacytidine Induces an Increase in rPRL Expression in a rPRL-Deficient Variant Isolated After EtMes Treatment. 5-Azacytidine can partially demethylate DNA *in vivo* because of its incorporation into daughter strands, during replication, where it cannot be methylated by virtue of having a nitrogen at the 5 position of the pyrimidine (23). The analogue has also been shown to promote the cytodifferentiation of cultured embryonic fibroblasts into muscle cells, chondrocytes, and adipocytes (25, 26). Moreover, it has also been shown to reactivate a genetically silent gene on the human X chromosome in somatic cell hybrids (27) as well as to activate the expression of dormant genes for metallothionein (28) and endogenous retrovirus (29). In the last two cases, activation of the genes was correlated with a decrease in their methylation.

To test whether the analogue could revert an EtMes-induced rPRL-deficient variant of GH_3 cells, we initially measured the effects of 5-azacytidine on rPRL production in a population of B_3 cells. These cells were cloned from a severely EtMes-mutagenized population of GH_3 cells and showed a decrease in rate of rPRL synthesis by a factor of 1/100 accompanied by a similar decline in the cytoplasmic level of pre-rPRL mRNA (12). When B_3 cells were treated with varying concentrations of 5-azacytidine for 2 days and assayed 13 days later for rPRL production as before, there was a dose-dependent increase in prolactin production, reaching a peak at 10–50 μM and declining thereafter (Fig. 1). The effects can be seen more clearly in rPRL immunoprecipitates (Fig. 1*B*; lanes 2–4). At 10 μM 5-azacytidine, B_3 cells were >80% viable as judged by their ability to attach and grow in culture flasks; a significant loss of viability (>50%) was seen at 50 μM and higher. The optimal dose, 10 μM, is comparable to that for the ability of the cytidine analogue to activate the metallothionein gene [*ca.* 8 μM (28)] but 3- to 5-fold higher than that required for promoting cytodifferentiation of embryonic fibroblasts (25, 26) or for activating the hypoxanthine phosphoribosyltransferase (HPRT) gene on the human X chromosome (27) and endogenous retroviral genes (29). This may reflect the fact that GH_3 cells are heteroploid (30).

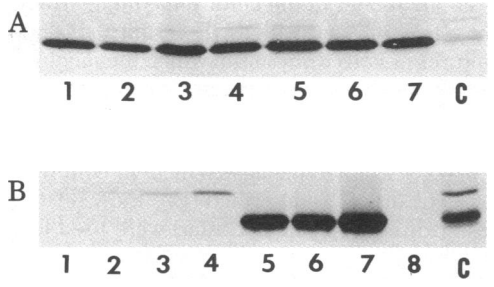

FIG. 1. Effects of 5-azacytidine on rPRL and rGH expression in a rPRL-deficient variant of GH_3 cells. The rPRL-deficient variant of GH_3 cells, termed B_3, was treated with various concentrations of 5-azacytidine (0–500 μM) for 48 hr in standard culture medium. After removal of the analogue, cells were incubated for 13 days before the assay for rPRL and rGH synthesis and secretion. (*A*) Lanes 1–7 are medium samples from cultures treated with 0, 1, 5, 10, 50, 100, and 500 μM 5-azacytidine, respectively. In this and subsequent figures, samples were loaded in proportion to the radioactivity incorporated by each clone. (*B*) Samples of media from cells not treated with inhibitor (lanes 2 and 5) or treated with 1 μM (lanes 3 and 6) or 10 μM 5-azacytidine (lanes 4 and 7) were immunoprecipitated with rabbit anti-rPRL (lanes 2–4) or rhesus monkey anti-rGH antiserum (lanes 5–7); lanes 1 and 8 are control immunoprecipitates with nonimmune rabbit and monkey sera, respectively. Lane C is medium from a dense wild-type GH_3 cell culture labeled for 5 hr with [^{14}C]leucine (12).

The magnitude of the effect of 5-azacytidine on rPRL and rGH expression in B_3 cells was quantitated by immunoprecipitating samples of the medium from treated and control cells with antisera specific to rPRL and rGH. Immunoprecipitated radioactivity in specific precipitates was corrected for nonspecific precipitation by control sera and normalized to the level of radioactivity incorporated by the separately treated cultures. At 10 μM, the optimal dose, the cytidine analogue increased rPRL synthesis and secretion about 3.5-fold, whereas it increased rGH synthesis and secretion by only 1.5-fold. Even at 1 μM, 5-azacytidine produced a detectable increase when assayed by the more sensitive method of gel electrophoresis and autoradiography (Fig. 1, lane 3). These results clearly show that 5-azacytidine substantially increases rPRL production in B_3 cells and are consistent with the idea that EtMes may generate the variants by promoting DNA methylation.

5-Azacytidine Reverts rPRL-Deficient B_3 Cells to rPRL-Expressing Cells at High Frequency. Because the effect of the inhibitor on B_3 cells was seen 13 days (or six to seven generations) after treatment, 5-azacytidine appeared to have induced a relatively stable change in rPRL synthesis. This might have occurred because the analogue uniformly increased rPRL expression 3- to 5-fold in all cells or reverted a subpopulation of B_3 cells to levels of rPRL expression near those observed for wild-type GH_3 cells. To test this, B_3 cells were treated or not with 10 μM 5-azacytidine for one generation (2 days) and grown for an additional 4 days in the absence of the analogue before they were cloned on agar surfaces; individual colonies were assayed for rPRL and rGH expression as before. Fig. 2 *B* and *C* illustrates that more than half of the colonies from the treated B_3 culture had reverted to detectable levels of rPRL expression whereas in the untreated population only one revertant was found (see below). The 5-azacytidine-induced revertants exhibited a high degree of stability in culture inasmuch as 2 generations had passed before cloning and an additional 20–30 generations had passed before assay on gels. It appears, therefore, that a major effect of the DNA methylation inhibitor is to convert a significant fraction (*ca.* 50%) of B_3 cells into stable rPRL producers. In this regard, Groudine *et al.* (29) estimated that about 50% of the dormant endogenous retroviral genes in MSB

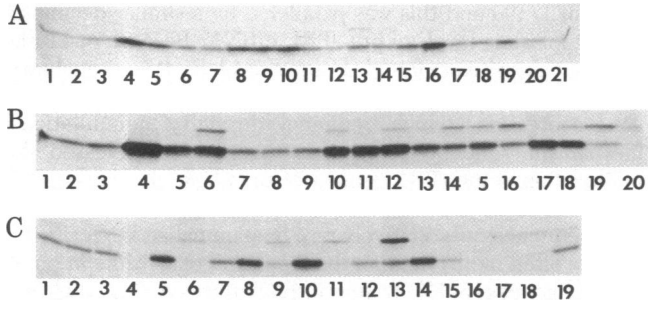

FIG. 2. Clonal analysis of rPRL-deficient B_3 cells after treatment with 5-azacytidine. B_3 cells were treated for 2 days with (*B* and *C*) or without (*A*) 10 μM 5-azacytidine, grown for 4 days, and then cloned on agar as before. rPRL and rGH synthesis were assayed among individual colonies as before. Numbers under the lanes refer to individual clones. Note that the level of [^{35}S]methionine incorporation was very low for colonies 4, 6, 11, and 16–18 in *C*.

chicken lymphocyte cells were activated by 5-azacytidine treatment under optimal conditions.

The sole revertant found in the untreated B_3 population (clone 13 in Fig. 2A) represents a "high-producing" revertant of B_3 cells. Previously, 1 weak revertant was found among 47 subclones of B_3 cells assayed for rPRL expression as described here, and no revertants were seen producing high levels of pre-rPRL mRNA among nearly 250 B_3 subclones assayed by colony hybridization procedures (12). Clone 13 simply may be a relatively rare isolate indicating a reversion frequency of B_3 cells near 1/320. Alternatively, the presence of clone 13 in the current stock of B_3 cells may indicate that rPRL revertants have increased to a frequency of 1/20 during the 30–40 generations that elapsed since the isolation and last clonal analysis of the B_3 line. Regardless, the isolation of clone 13 indicates that the effect of EtMes on GH_3 cells is not completely stable in the genetic sense (i.e., a classical mutational event). Furthermore, if EtMes acts by promoting specific DNA methylation with subsequent effects on rPRL expression, it would appear that GH_3 cells are unable to maintain the methylation change with 100% fidelity. It has been reported that cultured cells cannot maintain methylation of some sites with complete fidelity in foreign DNAs that were methylated *in vitro* and introduced into cells by DNA-mediated cell transformation (21, 22).

Two-dimensional gel analysis (data not shown) also revealed that one of the revertants induced by 5-azacytidine (clone 6, Fig. 2B) regained the expression of p21, a hormonally responsive protein in GH_3 cells (9, 10) whose expression is also absent from B_3 cells (12). The fact that p21 and rPRL corevert after 5-azacytidine treatment provides additional confirmation of the fact that their expression is tightly coupled in GH_3 cells, as was shown previously in a spontaneous revertant of an unstable rPRL-deficient variant (12). More recently, we have found that p21 is also expressed at high levels in pituitaries from female rats.

UV Irradiation Does Not Induce rPRL-Deficient Variants at High Frequency. To test whether the appearance of variants was limited to EtMes treatment, GH_3 cells were irradiated with UV light at a dose allowing 2–10% survival or were treated with EtMes as before (12), and individual colonies were assayed for rPRL and rGH expression. EtMes treatment resulted in survival of many individual clones that synthesized and secreted rPRL at substantially decreased or undetectable levels (especially clones 4, 7, 10, 12, and 17) (Fig. 3B). By contrast all 20 clones from the control population (Fig. 3A) or from the UV-irradiated population (Fig. 3C) synthesized and secreted rPRL and rGH at levels typical of wild-type GH_3 cells. To date, 40 clones from such irradiated cultures have been assayed and no variants have been found. It appears, therefore, that the process(es) leading to the appearance of the variants does not depend on mutagenesis *per se* but is relatively specific to EtMes treatment. Other alkylating agents have not yet been tested for their ability to mimic the action of EtMes on these cells.

The variation in rPRL and rGH expression among clones from control and UV-irradiated cultures evident in Fig. 3 is the result of cell density differences at the time of labeling. Cell density produces opposite effects on the production of the two proteins in pituitary tumor cells: high density stimulates rGH production but inhibits rPRL production (refs. 31 and 32; unpublished data). Clones 3 and 11 (Fig. 3C) were among the most dense at the time of assay and produced relatively low but readily detectable levels of rPRL whereas clone 6 was a sparse culture when assayed and produced low but detectable levels of rGH. Because such differences among clones fall within the range of cell density effects alone (12), such clones are not scored as deficient variants.

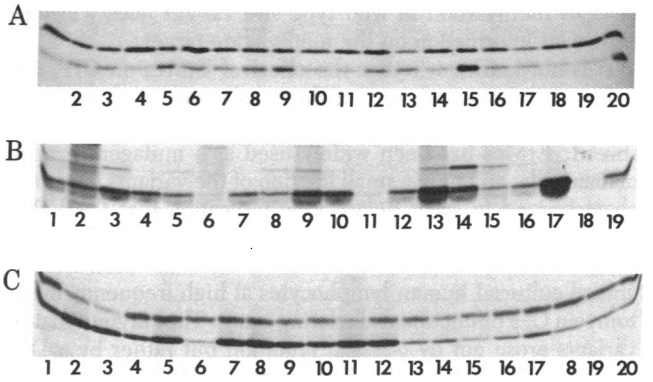

FIG. 3. Clonal analysis of the effects of EtMes and UV irradiation on rPRL and rGH expression in GH_3 cells. Wild-type GH_3 cells were treated with EtMes (300 μg/ml) for 24 hr (*B*) or irradiated with UV light at a dose of 200 ergs/mm^2 (*C*). Untreated, unirradiated wild-type cells served as a control (*A*). After mutagenesis, cells were cloned and individual colonies were assayed for rPRL and rGH synthesis and secretion. The incorporation level was very low for clones 6, 11, and 18 of *B*. Clones 9, 13, and 17 of *B* were very dense at the time of assay, leading to altered levels of rGH and rPRL as discussed in the text.

On the Action of EtMes in GH_3 Cells. The results reported here are consistent with the idea that the ability of EtMes to generate rPRL variants of GH_3 cells depends in part on methylation of cytosine residues in DNA. Because DNA is a major target of EtMes and other alkylating agents (see ref. 33), it is possible that ethyl adducts promote methylation of the rPRL gene directly, which subsequently leads to its inactivation. Alternatively, a gene regulating rPRL gene expression might be the methylation target. Several recent observations suggest one direct means by which this might occur. Both *in vivo* and *in vitro*, EtMes alkylates DNA in the N7 position of guanine with high efficiency [*ca.* 75% of all ethyl adducts (reviewed in ref. 33)]. Moller *et al.* (34) have shown that *in vitro* methylation of N7 guanine in poly(dG-dC)·poly(dG-dC) promotes the conversion of the right-handed helical B form of DNA to the left-handed Z form at physiological salt concentrations. Behe *et al.* (35, 36) obtained similar results when 5-methylcytosine replaced cytosine in the same model DNA. In their original description of Z-DNA, Wang *et al.* (37) proposed that the left-handed helix might be a better substrate for a DNA methylase because G·C base pairs are located on the outer convex surface of the Z helix where cytosine C5 would be more accessible than it would be in B-DNA. In this view, other DNA alkylating agents known to promote the transition of B-DNA to Z-DNA (38–41) might also generate a high frequency of rPRL-deficient variants in GH_3 cells. Up to 20% of the DNA adducts produced by EtMes treatment are ethyl phosphotriesters (30, 42). Thus, it is also possible that an ethyl adduct at guanine N7 or phosphodiester bonds, given their close proximity to cytosine C5 in CpG sequences in B-DNA, might be recognized by a DNA methylase as a fraudulent "hemimethylated" site, leading to methylation of CpG in the opposite strand. In this case, the size and chemical nature of the adduct would most likely have a strong influence on the ability of the enzyme to bind and function at the site.

It should be emphasized, however, that alkylating agents react with other bases and sites on guanine at lower efficiency (33, 42). RNA is also highly reactive yielding similar adducts. Thus, various mechanisms can explain the results.

The gene for rPRL (43) and full-length cDNAs to pre-PRL mRNA (44, 45) have been cloned and amplified in *Escherichia coli*. Probing the structure of the rPRL gene and the extent of

its CpG methylation in wild type and variant lines will yield important information on the methylation target.

It is not known whether the effect of EtMes on GH_3 cells reflects a unique property of these cells and, perhaps, of pituitary gene expression in general or whether it is more widespread. EtMes has been widely used as a mutagen in mammalian cells, and only a small fraction of the variants have been characterized in great detail. One indication that the EtMes effect may be more general is a recent report by Evans and Vijayalaxmi (46) on the induction of 8-azaguanine-resistant variants of cultured human lymphocytes at high frequency by mitomycin C, a difunctional alkylating agent. They argued that the variants arose not by classical mutation but rather by a DNA alkylation adduct in the hypoxanthine HPRT gene which blocked its transcription. Mitomycin C apparently facilitates B-DNA-to-Z-DNA transition at high efficiency (38) which, in light of our findings, suggests that it might also promote DNA methylation. Quite recently, glycosylases capable of removing 7-methylguanine from DNA have been detected and partially purified from bacterial and mammalian sources, indicating that mechanisms have evolved for repairing such alkylation products (47–49).

It is also notable that HPRT is X-linked in mammals and, as strongly implied by the results of Mohandas *et al.* (27), DNA methylation may underlie X inactivation. A class of 8-azaguanine resistant variants has been isolated from L cells at high frequency after mutagenesis with EtMes or nitrosoguanidine (50). The variants expressed a wide range of HPRT enzyme levels and reverted to wild type at high frequency (up to 10^{-2}). Mutants were also isolated at lower frequency, from L and CHO cells, in which HPRT enzyme activity was undetectable (51, 52). However, immunoreactive HPRT was absent from more than half of these mutants, suggesting that the $HPRT^-$ phenotype may have derived from an inhibition of HPRT gene expression rather than a structural gene mutation.

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