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Inactivation of a Transfected Gene in Human Fibroblasts Can Occur by Deletion, Amplification, Phenotypic Switching, or Methylation

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Plasmids containing the bacterial *gpt* gene under control of the simian virus 40 promoter were transfected into a simian virus 40-transformed human fibroblast line. Two transfectants, E2 and C10, which contain stably integrated single copies of the *gpt* gene, were isolated. These two lines produce Gpt⁻ variants spontaneously with a frequency of about 10^{-4}. We carried out a detailed molecular analysis of the spectrum of alterations which gave rise to the Gpt⁻ phenotype in these variants. DNA from 14 of 19 Gpt⁻ derivatives of one of the cell lines (E2) contains deletions or rearrangements of *gpt*-containing sequences. In four of the remaining five lines, the Gpt⁻ phenotype was correlated with reduced levels of expression rather than with changes in the gross structure of the *gpt* gene, and it was possible to reactivate the *gpt* gene. In one Gpt⁻ line, *gpt* mRNA was present at normal levels, but no active enzyme was produced. Spontaneous Gpt⁻ derivatives of the other cell line (C10) produced a completely different spectrum of alterations. Very few deletions were found, but several derivatives contained additional extrachromosomal *gpt* sequences, and, remarkably, in two other Gpt⁻ lines, *gpt*-containing sequences were amplified more than 100-fold. The phenotypes of the majority of the Gpt⁻ derivatives of C10 could be attributed to alterations in gene expression caused by methylation.

The ability to transfect bacterial or viral genes into mammalian cells has provided new approaches to the study of alterations in gene structure and expression in mammalian cells. Transfected DNA is known to integrate more or less at random into the host genome, and the stability and expression of the integrated genes may depend on the chromosomal environment at the site of integration. In many instances, the integrated genes are unstable, but in some cases, the exogenous genes are stably integrated. It is therefore possible to use the integrated genes as targets for mutation studies. The structure, stability, and inactivation of integrated genes have been the subjects of several studies, most of which have been confined to a single system, namely the herpes simplex virus thymidine kinase (*tk*) gene integrated into the genome of mouse Ltk⁻ cells. Inactivation of the integrated *tk* gene can occur in several different ways (4–6, 14, 18, 22).

The bacterial guanine phosphoribosyltransferase (*gpt*) gene is of more general use, as it is a dominant gene which can be transfected into almost any cell line (13), thereby broadening the host range in which studies of the structure, expression, and inactivation of integrated genes can be carried out. We introduced the *gpt* gene into a simian virus 40 (SV40)-transformed human fibroblast line and obtained two stable derivatives containing single copies of the *gpt* gene. We carried out a detailed analysis of the mechanism of inactivation of the gene in approximately 20 spontaneous Gpt⁻ derivatives of each line, and we report here on a whole spectrum of different alterations of the *gpt* gene including deletions and gene amplifications, as well as a variety of derivatives in which gene expression has been switched off in different ways.

MATERIALS AND METHODS

Cell culture. A hypoxanthine phosphoribosyltransferase (*hprt*⁻) derivative of the SV40-transformed human fibroblast line GM0637 (National Institute of General Medical Sciences Human Genetic Mutant Cell Repository, Camden, N.J.) was used in all experiments. This *hprt*⁻ derivative, designated line 1306, was a spontaneous mutant selected by growth in 5 μg of 6-thioguanine (TG) per ml. It contains a single integrated copy of SV40 and no detectable extrachromosomal SV40 sequences (unpublished observations).

All cells were grown in Eagle minimal essential medium (MEM) supplemented with 10% fetal calf serum. The medium for selection and maintenance of Gpt⁺ cells (MAX medium) contained (per milliliter) 25 μg of mycophenolic acid, 10 μg of xanthine, 15 μg of hypoxanthine, 0.2 μg of aminopterin, 5 μg of thymidine, 2.3 μg of deoxycytidine, and 5 μg of glycine (13). The selection medium for Gpt⁻ cells contained 5 to 25 μg of TG per ml.

Plasmids. The plasmids used for transfection experiments (Fig. 1) were pSV2gpt, containing the *gpt* gene under the control of the SV40 promoter, splice site, and poly(A) addition sites (13) and pCD11, in which the two plasmids pSV2gpt and pSV2neo (20) were linearized with *Eco*RI and ligated together. The *neo* and *gpt* genes are in the same orientation. The expression of the *neo* gene in mammalian cells confers resistance to the antibiotic G418. Plasmid pL10 (generously provided by P. Berg), in which a *Hin*dIII-*Bam*HI fragment containing the *gpt* gene has been cloned into the large *Hin*dIII-*Bam*HI fragment of pBR322, was used as a hybridization probe.

Transfection. The calcium phosphate procedure (8, 28) was used to transfect 1 μg of pSV2gpt or pCD11 (without carrier DNA) into 1306 cells. After 16 h of exposure to the

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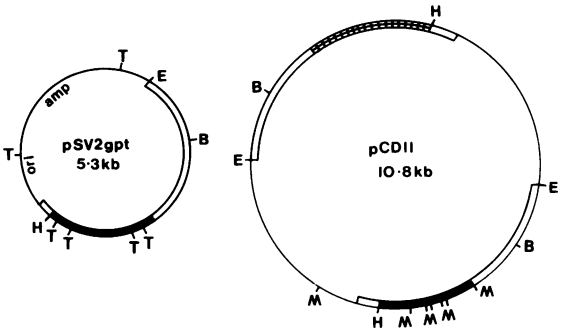

FIG. 1. Maps of plasmids pSV2gpt and pCD11. Sequences derived from pBR322 ——; SV40, ▭; *Escherichia coli* containing the *gpt* gene, ■; and transposon Tn*5* containing the *neo* gene, ▦. Abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; M, *Msp*I; T, *Taq*I (only *Msp*I sites within and immediately adjacent to the *gpt* gene in pCD11 are shown); ori, bacterial origin of replication; amp, β-lactamase (ampicillin resistance) gene.

precipitate, the medium was replaced with fresh MEM, and 24 h later, this medium was in turn replaced with MAX medium (for transfections with pSV2gpt) or MAX medium containing 100 μg of G418 (geneticin sulfate; GIBCO Laboratories) per ml (for transfections with pCD11). After 4 to 6 weeks, approximately 10 individual colonies from each transfection were picked and grown into mass culture. Two cell lines, E2 and C10, were used in further studies.

Selection of derivatives. C10 or E2 cells were grown for 1 week in MEM containing hypoxanthine, thymidine, and xanthine and then for at least 1 week in MEM; 10^5 cells were then plated in different concentrations of TG. A number of individual Gpt$^-$ colonies were picked and expanded in the presence of TG for further analysis.

The ability of some of these Gpt$^-$ derivatives to revert to the Gpt$^+$ phenotype was examined in two different ways. (i) Cells were grown for at least 1 month in MEM before being plated in MAX medium. (ii) Cells were treated for 48 h in MEM containing 5-azacytidine (0 to 10 μM) and then for 48 h in MEM alone and were then plated in MAX medium.

Southern analysis. DNA was extracted from cell pellets by standard proteinase K and phenol extraction procedures (12). DNA (25 μg) was digested for 4 h with 50 U of restriction enzymes and then electrophoresed in agarose gels. The DNA was transferred onto nitrocellulose or nylon membranes and hybridized either with pL10 nick translated to a specific activity of about 5×10^8 dpm/μg of DNA or with the *Hin*dIII-*Bam*HI *gpt* fragment of pL10 labeled by primer extension with Polymeraid (P and S Biochemicals, Liverpool, United Kingdom). Following hybridization at 42°C in solutions containing 50% formamide, the filters were subjected to several washes, the final wash being with 0.3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate at 65°C, and exposed to Fuji X-ray film.

RNA dot blots. Approximately 3×10^6 to 5×10^6 cells from two 9-cm dishes were scraped off the dishes into phosphate-buffered saline, centrifuged, and lysed as described by White and Bancroft (27). Cytoplasmic lysates corresponding to 3.75×10^5, 7.5×10^5, and 15.0×10^5 cells were filtered onto nitrocellulose (27) and hybridized as described above.

[^{14}C]guanine uptake. Various cell lines were seeded at a density of 10^5 cells on 5-cm dishes and radioactively labeled for 24 h with 0.05 μCi of [^{14}C]guanine (50 mCi/mmol) per ml. The cells were lysed on the dishes with 2% sodium dodecyl sulfate, and the incorporated radioactivity was measured on filter paper squares as described previously (11).

RESULTS

Establishment of cell lines with stably integrated single copies of the *gpt* gene. The plasmids pSV2gpt and pCD11 (which contains both the *gpt* and *neo* genes) (Fig. 1) were transfected into the *hprt*$^-$ SV40-transformed human fibroblast line 1306, and a number of Gpt$^+$ derivatives were isolated in MAX medium. When most of these Gpt$^+$ lines were plated in TG, Gpt$^-$ derivatives were obtained with a very high frequency (1 to 5%). These lines were considered too unstable for further study. Cells transfected with pCD11 were continuously selected for the presence of the *neo* gene in an attempt to generate derivatives with increased stability when selection for the *gpt* gene was removed. In most of these *neo*$^+$ derivatives, the *gpt* gene was also unstable. Two derivatives, designated C10 (from transfection with pSV2gpt) and E2 (from transfection with pCD11), were relatively stable. When initially tested, the frequency of production of Gpt$^-$ derivatives when plated in TG was about 1×10^{-4} to 2×10^{-4}. Over a subsequent 2-year period, there was a substantial fluctuation in this frequency, ranging from 4×10^{-5} to 1×10^{-3} in different experiments. Different treatment regimens were used that involved incubation for various periods in nonselective medium before plating in TG, but no consistent relationship was found between the treatment regimen and the frequency of production of Gpt$^-$ derivatives.

Restriction digests of DNA from C10 and E2 with a variety of enzymes revealed that a single copy of the plasmid had been integrated into the genome in each line. The structures of the integrated plasmids, as deduced from these digests, are shown in Fig. 2. In C10, the integration site is in the pBR322-derived sequences of pSV2gpt, with a deletion of approximately 500 base pairs of the plasmid in the region of the bacterial origin of replication. In E2, the integration site is in the bacterial sequences downstream from the *gpt* coding sequence (15).

Analysis of spontaneous Gpt$^-$ derivatives of E2. (i) [^{14}C]guanine uptake. *hprt*$^-$ cell lines are unable to incorporate exogenously supplied guanine into their DNA. The *gpt* gene can replace this function of the *hprt* gene, and measurement of guanine uptake provides a simple test for the activity of the *gpt* gene in *hprt*$^-$ cell lines (3). E2 can incorporate [^{14}C]guanine into nucleic acids, but none of the Gpt$^-$ derivatives showed any significant activity (Fig. 3a).

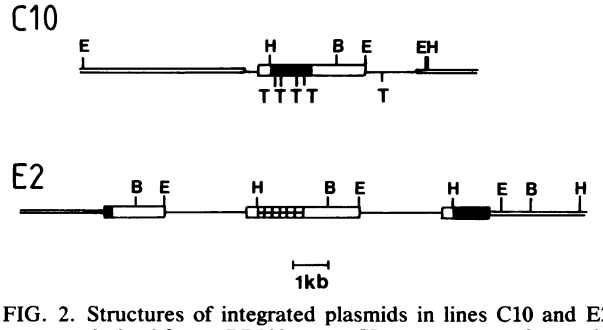

FIG. 2. Structures of integrated plasmids in lines C10 and E2. Sequences derived from pBR322, ——; SV40, ▭; *E. coli* containing the *gpt* gene, ■; *neo* gene, ▦; genomic flanking sequences, ═. Abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; T, *Taq*I.

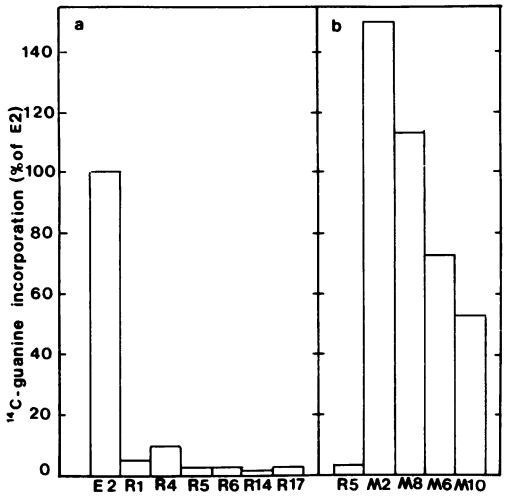

FIG. 3. Incorporation of [^{14}C]guanine into nucleic acids in E2 and derivatives. Incorporation over a 24-h period is expressed as a percentage of that in E2 cells for E2 and its TG-resistant derivatives (a) and for R5 and its azacytidine-reactivated derivatives (b).

(ii) Structure of the *gpt* gene. The DNA of E2 and 19 spontaneous Gpt$^-$ derivatives was digested with *Eco*RI and subjected to Southern analysis. Upon hybridization with the *gpt*-containing plasmid pL10, DNA from E2 revealed a 5.4-kilobase-pair (kb) band corresponding to the intact pSV2*neo* portion of pCD11 and a 4-kb band containing the *gpt* gene (Fig. 2; Fig. 4a and b, lanes E2). It can be seen from the examples in Fig. 4 that although in most of the Gpt$^-$ derivatives of E2, the 5.4-kb band containing the selected *neo* gene remained unaltered, the 4-kb *gpt*-containing band was no longer present, indicating that a deletion or rearrangement of *gpt*-containing sequences had occurred. In five lines, however (R1, R4, R5, R6, and R17), the *gpt*-containing band showed no detectable alteration.

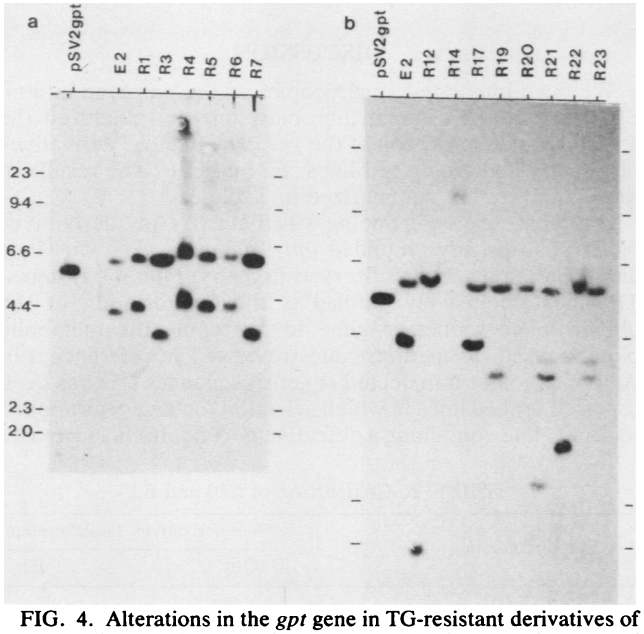

FIG. 4. Alterations in the *gpt* gene in TG-resistant derivatives of E2. DNA from various cell lines (25 μg) was digested with 50 U of *Eco*RI for 4 h, electrophoresed in 0.7% agarose gels, and transferred to nitrocellulose filters. These were hybridized with ^{32}P-labeled pL10. Panels a and b show results of separate experiments. The first lane in each contains 25 pg of *Eco*RI-digested pSV2gpt. The positions of *Hin*dIII fragments of lambda DNA are indicated.

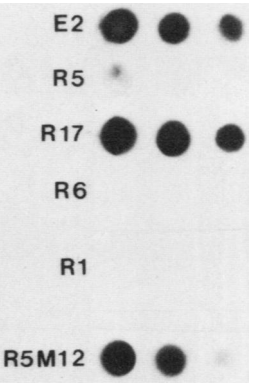

FIG. 5. Dot blots of *gpt* mRNA from various cell lines. Cytoplasmic lysates from (left to right) 15.0, 7.5, and 3.75 × 10^5 cells were spotted onto nitrocellulose filters and hybridized with ^{32}P-labeled pL10.

(iii) mRNA synthesis. The ability of Gpt$^-$ derivatives with unaltered sequences to synthesize *gpt*-specific RNA was analyzed by using dot blots of cytoplasmic extracts. A positive signal was obtained with E2, but very little or no *gpt*-specific RNA was detected in the derivatives R1, R5, R6 (Fig. 5), and R4 (data not shown), whereas in line R17, *gpt*-specific mRNA was present at similar levels to that in E2 (Fig. 5). These findings were confirmed by using Northern blots (unpublished observations) in which a *gpt*-specific mRNA was present in E2 and R17 but absent in R1, R5, and R6.

(iv) Reactivation of the *gpt* gene by phenotypic switching. Acquisition of the Gpt$^-$ phenotype could be brought about either by structural alterations (mutations) in the gene or its controlling sequences or by switching off gene expression by some other means. The five Gpt$^-$ lines which contained no gross alterations in the structure of *gpt* sequences were grown for 2 months in neutral (MEM) nonselective medium and then plated in MAX medium. Three of the lines (R1, R4, and R6) generated Gpt$^+$ lines at a frequency of greater than 10^{-2}. Lines R5 and R17 could not be reverted in this manner.

(v) Reactivation of the *gpt* gene by azacytidine treatment. Switching off of the expression of the *gpt* gene may have occurred as a consequence of methylation of CpG sites in the promoter region. Growth of cells in 5-azacytidine is known to cause undermethylation of the DNA, and this can lead to reactivation of genes which had been switched off by methylation (10, 16). Accordingly, Gpt$^-$ cells were treated with different concentrations of 5-azacytidine and then plated 2 days later in MAX medium for selection of Gpt$^+$ derivatives. With one of the Gpt$^-$ lines, R5, Gpt$^+$ revertants were obtained at a high frequency (approximately 2 × 10^{-3}) following azacytidine treatment. No such revertants were obtained under these conditions with the other four Gpt$^-$ lines with unaltered sequences.

A number of azacytidine-induced revertants of R5 were picked off and grown into mass culture. In these reactivated lines (designated R5M1 to R5M12), both the ability to incorporate [^{14}C]guanine into nucleic acids (Fig. 3b) and the synthesis of *gpt*-specific mRNA (Fig. 5) were restored.

Thus the 19 Gpt$^-$ derivatives of E2 consist of 14 with deletions or rearrangements, 1 azacytidine-revertible line, 3

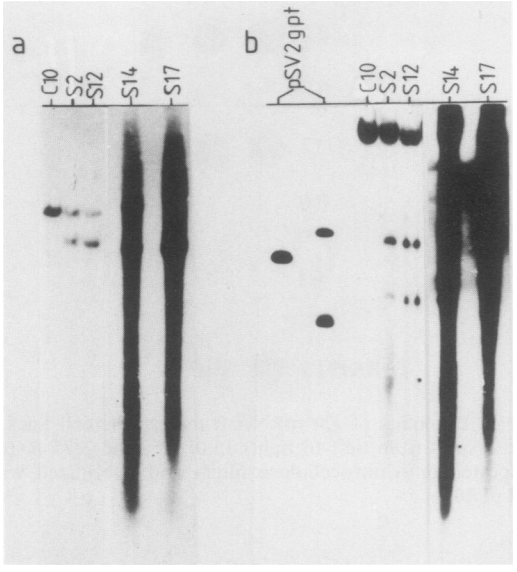

FIG. 6. Extrachromosomal and amplified *gpt* sequences in derivatives of C10. DNA from various lines (25 μg) was (a) digested with *Eco*RI or (b) not digested; it was then electrophoresed in 0.7% agarose gels, transferred to nylon filters, and hybridized with the ^{32}P-labeled *gpt* fragment. Lanes pSV2gpt in panel b contain 25 pg of *Eco*RI-digested and undigested pSV2gpt.

phenotypic switchers, and 1 line with no detected alteration. This line, R17, was the only derivative in which the *gpt* gene had no gross alteration in structure and could not be reactivated. Since *gpt*-specific mRNA but no functional protein was synthesized in this line, it may contain a point mutation in the *gpt* gene.

Analysis of spontaneous Gpt$^-$ derivatives of C10. Experiments similar to those described above carried out with Gpt$^-$ derivatives of line C10 selected in TG gave results which differed markedly from those with derivatives of E2. Digestion of C10 with *Eco*RI gave two fragments containing integrated sequences (Fig. 2), an 8-kb band containing the *gpt* gene (Fig. 6a, lane C10) and a 1.7-kb band containing bacterial plasmid sequences derived from pSV2gpt. Southern blots of EcoRI-digested DNA from Gpt$^-$ derivatives of C10 showed deletions in only 2 of 24 derivatives (results not shown). Fifteen derivatives showed the same digestion pattern as C10. In five derivatives (S2, S12, S15, S20, and S24), a further *gpt*-containing band of about 5.6 kb was seen on the autoradiograms of *Eco*RI-digested DNA (Fig. 6a, lanes S2 and S12; unpublished results). Extra bands were found even if the electrophoresis was carried out with undigested DNA (Fig. 6b, lanes S2 and S12), suggesting that these cell lines contain extrachromosomal molecules of *gpt*-containing DNA.

Our most remarkable finding was that in two further Gpt$^-$ lines, S14 and S17, there was an enormous amplification of *gpt*-containing sequences (Fig. 6a and b, lanes S14 and S17). Again, experiments with undigested DNA revealed that many of these amplified sequences were from extrachromosomal DNA (Fig. 6b).

We have investigated the ability to reactivate the *gpt* gene in Gpt$^-$ derivatives of C10 by growth in MAX medium either following treatment with 5-azacytidine or following a 1-month period in nonselective medium. None of 14 Gpt$^-$ lines examined was able to grow in MAX medium following 1 month of growth in the absence of selection. In contrast, 14 of 17 lines did revert to the Gpt$^+$ phenotype following azacytidine treatment. The reversion frequency ranged from 0.1 to 3% (Table 1). The azacytidine-revertible lines included four lines with an extrachromosomal *gpt*-containing band (see above) and one of the lines containing amplified *gpt* sequences (S14). The other amplified line (S17) was not azacytidine revertible. As with the E2 derivatives, none of 15 Gpt$^-$ derivatives showed significant uptake of [^{14}C]guanine. This activity was restored in several azacytidine-reactivated derivatives of S3, S9, S12, S14, and S23 (data not shown).

TABLE 1. Azacytidine revertibility of derivatives of C10

Cell line	Structural alteration[a]	Maximum frequency of reversion to Gpt
S1		
S2	E	2.6×10^{-2}
S3		5.7×10^{-3}
S4		7.7×10^{-3}
S5		–[b]
S6		1.0×10^{-2}
S7		0.9×10^{-3}
S9		1.4×10^{-2}
S10		1.1×10^{-2}
S11		5×10^{-3}
S12	E	1.2×10^{-2}
S13		3.7×10^{-3}
S14	A	8×10^{-4}
S17	A	
S20	E	3×10^{-2}
S23		1.4×10^{-2}
S24	E	3×10^{-3}

[a] E, Extrachromosomal band; A, amplification of *gpt* sequences. The absence of a letter signifies no gross structural alteration.

[b] One colony was obtained with this line. This gives a reversion frequency of about 3×10^{-5}, more than an order of magnitude lower than for other revertible lines.

DISCUSSION

We have integrated single copies of the *gpt* gene into an SV40-transformed human fibroblast line and analyzed the structure and expression of the *gpt* gene in Gpt$^-$ derivatives of two *gpt*-containing cell lines, E2 and C10. The results of these analyses are summarized in Table 2.

Our most surprising finding was that two Gpt$^-$ derivatives of C10 had greatly amplified *gpt* sequences (Fig. 6). Gene amplification has been observed in many different systems. The most intensively studied is the amplification of the dihydrofolate reductase gene to overcome the metabolic block caused by methotrexate (reviewed in reference 19). Amplification of transfected *tk* gene sequences (17) has been reported for cell lines in which selection for TK$^+$ was applied to a TK$^-$ line containing a defective *tk* gene. In this instance,

TABLE 2. Comparison of C10 and E2

Type of alteration	% of derivatives with alteration	
	C10[a]	E2
Deletion-rearrangement	8	74
Extra band	21	0
Amplification	8	0
Methylation	79	5
Phenotypic switching	0	15
No alteration detected	8	5

[a] Note that some derivatives of C10 had more than one type of alteration.

overproduction of the defective gene product was able to overcome the selective pressure. Similar results have been obtained with an *hprt*$^-$ mutant and revertants derived from it (7). In all these cases, the gene amplified was that for which selection was applied. Our results, however, are quite different. The *gpt* sequences were amplified in lines selected for the Gpt$^-$ phenotype from a Gpt$^+$ parent; i.e., there had been amplification of a gene against which selection was applied. There is no ready explanation for this phenomenon. The only other report of such a situation is that of a limited amplification of some *hprt* sequences in *hprt*$^-$ human lymphocytes from one individual described by Turner et al. (26).

In five further lines, we found extrachromosomal *gpt*-containing DNA which was not greatly amplified (Fig. 6). In all the lines containing extrachromosomal DNA, except for one of the amplified lines, the Gpt$^+$ phenotype could be restored following treatment with 5-azacytidine, suggesting that *gpt* sequences were methylated. It is thus possible that the sequences were methylated first and that extra sequences were then generated for reasons that remain obscure. We have found that lines containing extrachromosomal *gpt* sequences were not present at random at high frequency in the C10 population, since 10 subclones of C10 all had the same restriction patterns as the bulk population of C10 did (results not shown). The heterogeneous nature of the amplified sequences resembles that of amplified SV40 sequences produced on fusion of an SV40-transformed rat cell line with permissive monkey cells (1). We are at present analyzing the extrachromosomal and amplified DNA in our cell lines in more detail.

Another important observation is that the phenotype of many of the Gpt$^-$ lines resulted from switching off the expression of the *gpt* gene rather than from a genetic alteration. In one derivative of E2 and in most of the derivatives of C10, this inactivation resulted from methylation (Table 1). This conclusion is based on azacytidine-induced reactivation experiments. Although this evidence is indirect, the high frequency of azacytidine-induced reactivation and analysis of the expression of the *gpt* gene are entirely consistent with reactivation resulting from hypomethylation, probably of the SV40 promoter region of the *gpt* gene. Methylation of CpG residues has been shown to result in the inactivation of the expression of many endogenous mammalian genes and also the HSV-*tk* gene integrated into mouse cells (4, 5, 14, 22).

We have shown that methylation-independent phenotypic switching can occur with a dominant *gpt* gene in human fibroblasts. Similar observations have been reported in studies with transfected herpes simplex virus *tk* (6, 14, 18, 22) and *hprt* (9) genes. The mechanism of this phenomenon, which may in some cases involve alterations in chromatin structure (6), is not known.

The spectra of alterations in derivatives of E2 and C10 were completely different (summarized in Table 2). Although the majority of Gpt$^-$ derivatives of E2 were caused by deletions of *gpt* sequences, the majority of the C10 derivatives resulted from inactivation of the *gpt* gene by methylation. These differences can be attributed to a number of possible causes. (i) Since transfected DNA is thought to integrate into the genome at random, the sequences flanking the integrated DNA and the chromatin environment will be quite different. (ii) The integrated plasmids are different. Line C10 contains a copy of the plasmid pSV2gpt, whereas E2 was derived from transfection with pCD11 containing both pSV2gpt and pSV2neo sequences. pCD11 therefore has extensive regions of homology which may favor a particular type of alteration. (iii) The integration sites on the plasmids are different (Fig. 2). (iv) All experiments with line E2 were carried out with selection for the *neo* gene being maintained continuously. This may also place constraints on the permissible types of alteration in this line. Thus, for example, amplification of the inserted sequences may give rise to overproduction of the *neo* gene product, which may be toxic for the cells.

Chinese hamster cell lines containing integrated *gpt* genes have been used for the study of induced mutation by Thacker et al. (23) and by Tindall and co-workers (21, 24, 25). Tindall et al. used a variety of different agents to induce mutations in a Chinese hamster ovary cell line containing a single copy of the *gpt* gene (21). The relative distributions of deletions and mutations without detectable alterations in the *gpt* gene following treatment with different mutagens was dependent on the nature of the mutagen and was similar but not identical to the distributions of these types of alterations in the endogenous *hprt* gene in the parental Chinese hamster ovary line. None of the derivatives was analyzed for the possibility of inactivation of expression of the *gpt* gene. Our results suggest that caution must be exercised in the use of integrated exogenous genes as mutation systems, since inactivation of gene expression may account for a substantial proportion of putative mutants in which no gross alteration of gene structure can be detected. Such lack of detectable alterations cannot be regarded as sufficient evidence for the existence of point mutations. The possibility that alterations in gene expression account for a significant proportion of apparent mutations in endogenous genes must also be considered. For example, in a recent study, Brown et al. (2) measured the revertibility of a number of *hprt*$^-$ mutant hamster V79 lines. A number of phenotypes were observed, some of which were compatible with alterations in gene expression.

In summary, we have demonstrated that gene inactivation in human cells can be mediated by a variety of different mechanisms. Our cell lines may provide excellent systems with which to study the effect of mutagens on gene expression and gene amplification. Current studies are aimed in this direction.

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