High-Frequency Changes in Transcriptional Activity in Polyomavirus-Transformed Cell Lines

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We applied the Luria and Delbruck fluctuation test to analyze high-frequency changes in the phenotype of rat cells transformed by a plasmid carrying the polyomavirus middle T (*pmt*) gene. All of the transformed cell lines analyzed were capable of switching to the normal state with rates ranging from 10^{-3} to 10^{-2} per cell per generation. Analysis of both middle T antigen and middle T transcripts indicated that the reversion occurred by a mechanism involving a transcriptional block of the *pmt* locus. Cell lines containing two separate loci reverted with a lower rate, suggesting that phenotypic switching in these cells involved two independent events affecting each locus. The flat revertants mutated to the transformed state with rates in the range of 10^{-5} to 5×10^{-5} per cell per generation. To determine whether changes in *pmt* expression would affect neighboring sequences, we transfected a hybrid plasmid carrying *pmt* linked to the *neo* marker and selected either for morphological transformants or for G418-resistant cells. Although their coordinate regulation was not absolute, both genes were usually subject to the same changes, reflected by loss and reacquisition of transcriptional activity.

The alteration of gene expression in mammalian cells is thought to occur generally by mechanisms involving changes in the structure or activity of the promoter. However, other levels of control appear to be involved as well. For example, the expression of retroviral genes, integrated at random in the host chromosome, can be subject to cellular regulatory mechanisms so that the extent of expression results from a constant interplay between two different modes of regulation: the promoter activity and the local cellular controlling elements (13). In our laboratory, we have been interested in the expression of the polyomavirus middle T (*pmt*) antigen gene stably integrated into the genome of rat cells. We have shown previously that in the established FR3T3 cell line transfected with the polyomavirus genome, acquisition of the fully transformed phenotype correlates with effective expression of the polyomavirus oncogene (1, 7). Flat cells carrying integrated copies of *pmt* are not resistant to transformation because they can be readily transformed by retransfection with *pmt* (1). Furthermore, the flat cells are converted spontaneously to the transformed state with a rate of 2×10^{-5} mutations or spontaneous events per cell per generation. The transformed variants contain elevated levels of both middle T antigen and middle T transcripts, which suggests that they arise as a consequence of transcriptional activation. Very little is known about the mechanism underlying these events and the cellular signals that result in the activation of *pmt* expression. In this work we show that most cell lines transformed by the *pmt* oncogene are capable of switching to the normal phenotype at high frequencies by a mechanism involving a transcriptional block of the *pmt* locus. Furthermore, by cotransfecting the *neo* gene with *pmt*, we have been able to analyze the fate of an adjacent marker. We show that the transfected genes are usually regulated coordinately and that they are subject to high-frequency changes, reflected by loss and reacquisition of transcriptional activity.

MATERIALS AND METHODS

Plasmids. pMT3 carries the *pmt* gene. This recombinant was obtained by deleting two *Hind*III fragments from pPyMT1 (20). pSV2*neo* is a plasmid expressing *neo*, a dominant selection marker (18). *pneo*-MT3 (Fig. 1) was constructed by inserting the *Bam*HI-*Eco*RI fragment of pPyMT1 (20) into pSV2*neo*.

Cells and culture. All cells were grown at 37°C in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. Recombinant plasmids were isolated from bacteria and purified by CsCl density gradient centrifugation, and the closed circular DNA was transfected into monolayers of FR3T3 cells (17) by using the calcium chloride-dimethyl sulfoxide procedure (19). Transformants were scored as dense foci after 2 weeks of incubation. This assay typically yielded transformation frequencies of about 50 transformants per μ g of cloned wild-type genomic DNA per 5×10^5 cells and about five times less with pMT3 (*pmt* alone). Colonies of transformed cells were picked, established into cell lines, and subcloned by several rounds of single-cell cloning in 6-mm Linbro microplates (Flow). To apply the Neo selection, the cells were plated at 20 to 30% confluence and, after 18 h, G418 was added at a concentration of 400 μ g/ml. The medium plus drug was changed every 5 days. Colonies were first detected after 7 to 10 days in the selective medium, and 2 to 3 weeks later, independent colonies were picked, transferred into 15-mm Linbro microplates, and grown at least once in medium containing G418 (400 μ g/ml).

Measurements of mutation rates. Analyses were based upon the fluctuation test of Luria and Delbruck (11). Replicate cultures of untransformed cells carrying transcriptionally inactive copies of *pmt* were initiated by seeding a small number of cells (less than 50/cm²) in 6-mm Linbro microplates and allowing them to grow to confluence at 37°C. The rate of mutations leading to the transformed phenotype was estimated by taking into account only the fraction of replicate cultures without transformed cells (7). To determine the

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FIG. 1. Structure of *pneo-MT3*, a hybrid plasmid carrying both the *neo* and *pmt* genes. To link *pmt* to *neo*, pPyMT1 was cleaved by *Bam*HI plus *Eco*RI (nucleotide 1560), and the fragment carrying *pmt* was inserted between the *Bam*HI and *Eco*RI sites of pSV2neo. The position of the deleted intron in *pmt* is indicated. Abbreviations: B, *Bam*HI; Pv, *Pvu*II; R, *Eco*RI; ori, origin.

rate of reversion to the normal phenotype, the transformed cell lines were subcloned, seeded at very low densities (5 to 10 cells per cm²), and allowed to expand into colonies. Colonies of various sizes were isolated, dispersed into single cells with trypsin, and replated at low densities in 10-cm dishes so as to allow the identification of flat revertants in the cell population. The population sizes (N_f) were determined by counting either the number of cells in original colonies or, in case of large populations, the number of colonies developing from single cells after their dispersion in culture. Care was taken to ensure that the plating efficiency was close to 100%. The rate of mutations leading to the flat phenotype was evaluated by taking into account only the fraction of replicate cultures without flat revertants. This approach eliminates the possibility of scoring two or more revertants originating from a single mutational event. In an unsynchronized culture of growing cells, the total number of generations that has occurred is given by the expression $(N_f - N_i)/\ln 2$, where N_f and N_i are the final and initial numbers of cells. The average number of mutants per culture, m , can be estimated as $-\ln P_0$, where P_0 is the probability that no mutational event will occur. The mutation rate is given by the expression $a = (-\ln P_0 \cdot \ln 2)/(N_f - N_i)$ per cell per generation.

Analysis of middle T antigen. After reaching confluence, the cells were washed twice with phosphate-buffered saline, and the proteins were extracted and immunoprecipitated with an anti-polyomavirus T protein serum as described previously (9). The kinase activity associated with the middle T antigen was assayed as described by Schaffhausen and Benjamin (16).

RNA isolation. The procedure for the isolation of mRNA has been described elsewhere (7). Poly(A)⁺ RNA was isolated by oligo(dT)-cellulose chromatography (12) and precipitated with sodium acetate (final concentration of 0.3 M) and 2.5 volumes of ethanol at -20°C. The yield from 10⁸ cells varied between 10 and 20 µg of poly(A)⁺ RNA.

RESULTS

Isolation of flat revertants and spontaneous retransformants. In an attempt to evaluate reversion rates, we undertook to isolate flat revertants from various polyomavirus-transformed cell lines in the absence of any killing agent. We reasoned that revertant cells, appearing at relatively high frequencies, could be detected by examining the morphology of every clone growing from single cells seeded in sparse culture. The cell lines used are listed in Table 1. They were obtained by transforming FR3T3 cells with the *pmt* gene

transfected alone or together with the *neo* marker. Surprisingly, revertants could be detected in cell populations as small as a few hundred cells, indicating that reversion from a transformed to a normal state occurred at a higher rate than was previously reported (13, 21). The revertant clones had a morphology characteristic of untransformed cells. They grew more slowly than their transformed counterparts and exhibited contact inhibition. Some of them appeared to be highly unstable, as they gave rise to transformed cells before they could be isolated and expanded into cell lines (e.g., less than 1,000 cell divisions). This behavior seemed to be characteristic of revertant clones originating from highly transformed, tumorigenic cell lines. By contrast, partial transformants, i.e., cell lines with less malignant phenotypes, produced revertants that could easily be expanded into cell lines. However, when these cell lines were passaged in culture, they too gave rise to foci of transformed cells. Several revertants and spontaneous retransformants were assessed for a number of biological properties associated with transformation. Unlike the parental transformed cell lines and the retransformants, none of six revertants tested reached high saturation densities, grew in soft agar, or developed a tumor when 50,000 cells were inoculated subcutaneously into nude mice (data not shown).

Luria-Delbrück fluctuation analysis. We applied the Luria-Delbrück fluctuation analysis to determine whether both flat revertants and retransformants arose by stochastic processes. In this approach, the ratio of the variance to the mean should be much larger for the clonal sampling than for the replica sampling of individual clones. Such a condition was met statistically for the various cell lines analyzed (see Table 3, footnote a). To determine the rate of reversion to the flat phenotype, parallel clonal populations were grown to a sufficiently small size so that no variants would be observed in a significant proportion of populations. By growing

TABLE 1. Luria-Delbrück fluctuation test for spontaneous reversion to the flat phenotype^a

Cell line	No. of replica cultures	Culture size (N_f)	Proportion of cultures without revertants (P_0)	Reversion rate (per cell per generation)
8-2C1	6	319 ± 48	2/6 (0.33)	2.3×10^{-3}
3-9	5	338 ± 37	1/5 (0.2)	3.3×10^{-3}
3-11	6	143 ± 50	1/6 (0.17)	8.7×10^{-3}
d/8MT3-4	6	38 ± 25	4/6 (0.67)	7.5×10^{-3}
	3	121 ± 26	1/3 (0.33)	6.3×10^{-3}
d/8MT3-5	6	203 ± 63	4/6 (0.67)	1.4×10^{-3}
d/8MT3-7	10	285 ± 88	5/10 (0.5)	1.7×10^{-3}
d/8MT3-23	7	326 ± 56	2/7 (0.29)	2.7×10^{-3}
d/8MT3-32	7	175 ± 43	5/7 (0.71)	1.3×10^{-3}
d/8MT3-34	9	214 ± 49	7/9 (0.78)	0.8×10^{-3}
d/8MT3-37	7	62 ± 37	3/7 (0.43)	9.4×10^{-3}
neoMT-8	4	67 ± 23	2/4 (0.5)	7.2×10^{-3}
3-10	10	172 ± 56	6/10 (0.6)	2.1×10^{-3}
3-10neoMT1	9	308 ± 142	8/9 (0.89)	2.6×10^{-4}
3-10neoMT2	9	398 ± 227	7/9 (0.78)	4.3×10^{-4}
3-10neoMT3	9	251 ± 134	9/9 (1)	$<3.2 \times 10^{-4}$
3-10neoMT4	9	462 ± 125	9/9 (1)	$<1.7 \times 10^{-4}$
3-10neo	3	186 ± 111	2/3 (0.67)	1.5×10^{-3}

^a Transformed cell lines were subcloned and seeded at very low densities (5 to 10 cells per cm²) in 6-cm dishes. Colonies of various sizes were isolated, dispersed into single cells with trypsin, and replated at low densities in 10-cm dishes so as to allow the identification of flat revertants in the cell population. The reversion rate is given by the expression $(-\ln P_0 \cdot \ln 2)/N_f$ per cell generation, where N_f is the average number of colonies in replica cultures. Cell lines of the 3-10neoMT series were obtained by retransfection of 3-10 with *pneo-MT3*. 3-10neo was obtained by retransfecting 3-10 with pSV2neo.

subclones for appropriate numbers of generations, we determined that the cell lines yielded flat revertants with rates in the range of 10^{-3} to 10^{-2} per cell per generation (Table 1). The revertants that could be propagated in culture (see above) maintained a stable saturation density for several weeks. However, like various flat cell lines carrying transcriptionally inactive copies of the *pmt* gene (1, 7), they eventually yielded foci of transformed cells that overgrew the flat monolayer and reached high saturation densities (see, for example, Fig. 4). Based upon a fluctuation analysis, the revertant cell lines mutated to the transformed state with rates ranging from 10^{-5} to 5×10^{-5} per cell per generation (Table 2).

Analysis of *pmt* expression. Previous studies from this laboratory reported that there is a very good correlation between the phenotype of middle T-transformed cells and their level of *pmt* expression (1, 7). Therefore, we wished to see whether there were any differences in *pmt* expression that could account for the revertant phenotype. All of the revertant cell lines analyzed lost the ability to express the middle T antigen, as revealed by the kinase assay (Fig. 2). By contrast, all of the retransformants exhibited a kinase reaction similar to that of the transformed parental cell line. Likewise, the flat revertants did not contain any detectable middle T mRNA, whereas the retransformants produced at least the same amount of RNA as did the parental cell line (Fig. 3). These results indicated that loss and reacquisition of transcriptional activity were responsible for the phenotype variation observed in polyomavirus transformants.

Analysis of integration sites. We analyzed by Southern blotting the arrangement of the plasmid sequences within the DNA of about 15 different cell lines. Details will be presented elsewhere (L. Bouchard, manuscript in preparation). No free copies of recombinant plasmids (less than 0.2 copy per cell) were detected. Only two of the cell lines analyzed contained a single insertion of *pmt*. The other lines contained several inserts of the transfected plasmid, some of which were in head-to-tail arrangements. No correlation was found between the number or arrangement of integrated copies and other parameters such as cellular phenotype, level of *pmt* transcription, and frequency of phenotypic switching. Two lines of evidence suggested that integration of the transfected DNA occurred at a unique chromosomal site. First, if the *pmt* inserts were scattered throughout the whole genome, it is unlikely that all of the copies would be transcriptionally inactive in flat revertants. Second, it was observed previously that in a cell line containing 35 to 40

FIG. 2. Expression of middle T antigen in revertant cell lines and in their retransformants. Lanes: 3-9, Fully transformed cell line established by transfection of FR3T3 with pMT3 (*pmt* alone); neoMT-8, morphologically transformed cell line isolated as a colony of G418-resistant cells after transfection of FR3T3 with *pneo*-MT3; R5, R11, R12, and R6, flat revertant cell lines isolated from neoMT-8; R5T, R11T, R12T, and R6T, spontaneous retransformants. The kinase activity associated with the middle T antigen was assayed as described previously (16). Reactions were performed on 2×10^6 cells as described (7). The position of the middle T antigen at 56,000 daltons is indicated.

copies of pMT3, the DNA sustained a major deletion of the insert, leaving only one intact copy of the integrated plasmid (7). This observation is consistent with the hypothesis that the *pmt* copies are clustered in a single chromosomal site. To prove this point directly, we attempted to demonstrate that the frequency of reversion to the flat phenotype should be much smaller in cell lines containing multiple sites of integration. To do so, one of the *pmt* transformants, cell line 3-10 (Table 1), was retransfected with *pneo*-MT3, a plasmid carrying both *pmt* and *neo* (Fig. 1), in such a way that virtually all of the clones isolated as G418-resistant colonies carried a second *pmt* insert in a different chromosomal

TABLE 2. Mutation rate of flat revertant cell lines to the transformed phenotype^a

Cell line	No. of replicate cultures	No. of cultures without foci	P_0	Mutation rate (10^{-5})
R3	83	34	0.41	3.1
R5	68	18	0.26	4.6
R6	84	21	0.25	4.7
R8	78	26	0.33	3.8
R11	56	36	0.64	1.5
R12	83	37	0.46	2.7

^a Cells (about two) were seeded in 6-mm microplates. At confluence, the cultures contained an average of about 18,000 cells per well. The foci appeared after 5 to 6 weeks. The number of cultures without foci were counted after 7 weeks. P_0 is given by the proportion of experimental replicas lacking foci. The mutation rate is given by the expression $(-\ln P_0 \cdot \ln 2) / (N_f - N_i)$ per cell per generation, where N_f and N_i are the final and initial numbers of cells.

FIG. 3. Expression of middle T and *neo* transcripts in revertant cell lines and in their retransformants. Poly(A)⁺ RNA was fractionated on formaldehyde-agarose gels according to standard procedures (12). RNA blots were hybridized with middle T (left panel) and *neo* (right panel) probes. The middle T probe was obtained by nick translation of the *Bam*HI-*Eco*RI fragment of *pneo*-MT3 (Fig. 1). The *neo* probe was obtained by nick translation of the *Pvu*II-*Bam*HI fragment of *pneo*-MT3. Arrows indicate the positions of the 28S and 18S rRNAs.

FIG. 4. Phenotype of *neoMT-8*-derived cell lines. the parental cell line was isolated as a colony of G418-resistant cells after transfection with *pneo-MT3*. R5T, R11T, and R12T are spontaneous retransformants from flat revertants R5, R11, and R12, respectively. Resistance to G418 (NEO^R) was established using 400 μ g of G418 per ml. Magnification, $\times 133$.

location. We showed previously that under these conditions of retransfection, about half of the G418-resistant colonies expressed the cotransfected *pmt* gene (1). Furthermore, as 3-10, the recipient cell line, expressed relatively low levels of *pmt* (7), colonies with an additional (and transcriptionally active) *pmt* insertion were expected to appear more transformed in culture. Four such colonies, designated 3-10neoMT1 to 4 (Table 1), were picked for further analysis. Although these colonies were not characterized in terms of arrangement and chromosomal location of their additional integration site, it is clear that they reverted with a rate substantially lower than that of the parental cell line. By contrast, introduction of *neo* alone into the line did not modify its reversion rate. We propose that reversion of the 3-10neoMT cell lines involves two independent events, affecting each of the two different sites of *pmt* integration. Furthermore, although most of the cell lines carried multiple copies of *pmt*, integration of the transfected DNA occurred likely at a unique chromosomal site.

Modulation of *pmt* and *neo* expression. It was shown previously that genes introduced into cells by ligated cotransfection could be regulated coordinately and that the unit of this regulated expression could be at least 20 kilobases long (15). It was of interest to determine whether

changes in *pmt* expression affected neighboring gene sequences. For these studies we examined the activity of the *neo* marker that was cotransfected with *pmt* in FR3T3 cells. Transfection of *pneo-MT3*, the plasmid carrying both *neo* and *pmt*, did not always result in expression of both genes. When we selected for *neo* expression, only half of the G418-resistant colonies (36 of 72) exhibited a transformed phenotype and expressed the middle T antigen-associated kinase activity (data not shown). When we selected for morphological transformation in the absence of G418 selection, about half of the clones (12 of 27) expressed resistance to G418. *neoMT-8* was one of the cell lines isolated as a colony of G418-resistant cells exhibiting a transformed phenotype. It contained five copies of plasmid *pneo-MT3* (not shown) and yielded flat revertants with a rate comparable to that of other cell lines transformed by *pmt* alone (Table 1). Surprisingly, when the revertants were tested for growth in G418, all of them had lost the resistance. Six revertants were grown to confluence, and spontaneous retransformants were isolated and subcloned. The morphology of representative cell lines is shown in Fig. 4. All of the six retransformants tested were capable of growing in the presence of F418, indicating that the *neo* and *pmt* genes were regulated coordinately.

Although the retransformants expressed the *neo* gene at the time of focus selection, the maintenance of *neo* expression in the absence of G418 was somewhat variable among the various cell lines. For example, after 18 to 20 passages without G418, only two of six retransformants and the parental line had retained over 80% of the resistance to G418, whereas one of the retransformants (R3T) had a resistance of the order of 5%. Northern blot (RNA blot) analyses indicated that the loss of G418 resistance corresponded to a decrease in the level of the *neo* RNA isolated from the cell line (data not shown). This suggested that the coordinate regulation of the *pmt* and *neo* genes was not absolute but that *neo* transcription could be inactivated in subpopulations of cells even though these cells maintained a high level of *pmt* expression. We were interested to see whether *pmt* transcription could, like that of *neo*, be modulated independently. To this end, we grew the flat revertants to various population sizes and selected for G418-resistant cells. The cell lines yielded Neo^r cells in a stochastic manner with mutation rates ranging from 0.2×10^{-5} to 1.0×10^{-5} per cell per generation, i.e., about five times less than for the conversion of these cells to the transformed phenotype (Table 3). This difference was probably related to the difference in the selection process. Interestingly, about half of the Neo^r colonies did not express the transformed phenotype (not shown), suggesting that in these clones the spontaneous activation of *neo* was not matched by a similar activation of *pmt*.

DISCUSSION

Revertant cell lines having the growth properties of normal cells have been isolated from a variety of virus-transformed cell lines. In general, revertants can arise by a loss of the viral genome, a mutation or deletion in the transforming gene, a mutation in a cellular gene required for expression of the transformed phenotype, or a transcriptional block of the viral transforming gene. The revertants isolated in this work belong to the last category, i.e., they contain a complete viral genome but are unable to synthesize stable mRNA owing to a defect in cellular functions. This type of revertant is not unique to polyomavirus. A similar mechanism has been implicated in the generation of revertants isolated from rat fibroblasts infected with Fujinami sarcoma virus (13). We determined that the middle T transformants containing, presumably, a single integration site reverted to a normal phenotype with rates in the range of 10^{-3} to 10^{-2} per cell per generation. These rates are substantially higher than those previously reported (13, 21). The reasons for this are unclear but may involve the nature of the mechanisms of reversion or differences in the transformation procedures.

Possibly, two different mechanisms or levels of control can be considered to account for the transcriptional inactivation of the transfected genes. The first is illustrated by experiments using a hybrid plasmid encoding both *neo* and *pmt*. Transfection of this plasmid into FR3T3 cells does not always result in expression of both genes. The cotransfected marker is expressed only half of the time, regardless of the selection applied. We propose that in this case integration of the plasmid has occurred in a region of the chromatin that is compatible with efficient gene expression but which does not allow transcription from the cotransfected marker. The nature of the suppression is not known. It could involve epigenetic events such as transcriptional interference (2, 10), independent upstream and downstream suppression (4, 5),

TABLE 3. Mutation rate of flat revertants (Neo^s) to the Neo^r phenotype^a

Cell line	No. of replica cultures	Culture size (N_f)	Proportion of cultures without Neo ^r colonies (P_0)	Mutation rate (per cell per generation) (10^{-5})
R3	7	50,000 $\pm$ 18,000	4/7 (0.57)	0.7
	4	138,000 $\pm$ 20,000	1/4 (0.25)	0.7
R5	7	196,000 $\pm$ 48,000	1/7 (0.14)	0.7
R6	5	100,000 $\pm$ 47,000	1/5 (0.20)	1.1
	5	251,000 $\pm$ 46,000	0/5 (0.00)	
R8	10	95,000 $\pm$ 49,500	7/10 (0.70)	0.2
R11	5	59,000 $\pm$ 24,000	3/5 (0.60)	0.6
	7	172,000 $\pm$ 60,000	2/7 (0.29)	0.5
R12	6	62,500 $\pm$ 25,000	6/6 (1.00)	
	6	140,000 $\pm$ 30,000	5/6 (0.83)	0.1

^a Parallel clonal populations of flat revertants were grown to various sizes and treated with G418 at a concentration of 400 μ g/ml. The culture size (N_f) is the number of cells in replica cultures at the time of G418 selection. Colonies of G418-resistant cells were counted after 2 to 3 weeks of selection. P_0 is given by the proportion of replica cultures lacking Neo^r colonies. The mutation rate is given by the expression $(-\ln P_0 \cdot \ln 2)/N_f$ per cell per generation. As exemplified hereafter for the R5 cell line, the variance/mean ratio is much larger for the clonal sampling than for the replica sampling of individual clones. Clonal sampling, R5: Mean number of Neo^r colonies per replica, 72.4; variance, 8.975; variance/mean, 124. Replica sampling, R5: Number of samples, 10; culture size, 20,000; mean number of Neo^r colonies, 71.6; variance, 69; variance/mean, 0.96.

or even a totally different mechanism, since the lack of transcriptional activity in our system is not necessarily caused by expression from another nearby promoter. A second level of control, overriding eventually the first one, is a chromosomal change affecting transcriptional activity over large stretches of DNA. It too could possibly involve epigenetic events. A recent study has shown that inactivation of a transfected bacterial *gpt* gene in human fibroblasts can occur, in some cases, by methylation (6). Experiments in progress in this laboratory are showing that the ability of middle T transformants to revert at high rates is dependent upon the presence of CpG clusters in the vicinity of the *pmt* gene (unpublished data).

We have shown that the revertant cell lines mutate to the transformed state with rates ranging from 10^{-5} to 5×10^{-5} per cell per generation. Similar rates have been observed previously for the generation of spontaneous transformants in various cell lines carrying transcriptionally inactive *pmt* (1, 7), as well as in flat revertants arising from a frameshift at a mutational hotspot in the polyomavirus early region (21). In the latter case, the spontaneous transformation is due, at least in part, to the ability to correct precisely the revertant mutation. In the former case, the mechanism underlying the activation of *pmt* expression is not fully understood. It could involve epigenetic events or specific genetic events operating at high rates. The rate of 10^{-5} is significantly higher than that anticipated for a classical mutation. It is, however, within the range of the rates measured for genetic events associated with gene amplification and rearrangement. Green et al. (8) have observed frequent rearrangements immediately upstream of an intact provirus in Rous sarcoma virus-transformed rat cells. Such rearrangements occur during or soon after proviral integration and are thought to promote proviral expression. Furthermore, we have also observed frequent rearrangements in the *pmt* inserts in transformed variants occurring as a result of *pmt* activation (7; L. Bouchard, unpublished data). Thus, the activation of *pmt* expression in flat revertants could involve events such

as gene amplification or excision within or outside the viral insert.

Our previous work has shown that, in established cell lines transfected with *pmt*, acquisition of the fully transformed state correlated with effective expression of the middle T protein (1, 7). This is consistent with a model in which transformation is not an all-or-none phenomenon but, as previously shown for a retroviral oncogene (13, 14), is a function of the dosage of the oncogene RNA. It has been suggested that the fate of foreign DNA in mammalian cells is dictated by the location of its integration (3). Although the evidence is limited, we believe that the incidence of getting transcriptionally active or inactive *pmt* in established rat cells is influenced by the integration site and that introduced genes can be subject to high-frequency changes in expression. Similar results have been obtained by another group (15), which has shown that transfected thymidine kinase and globin genes can be regulated coordinately and that the unit of this regulated expression can be at least 20 kilobases long. Thus, besides *cis*- and *trans*-acting elements that have been identified for efficient gene expression in mammalian cells, other levels of control appear to be involved as well in the phenotypic modulation of cells carrying an oncogene. We tentatively conclude that the modulations of *pmt* expression in our polyomavirus transformants are associated with alterations in chromatin structure and that the *pmt* inserts are subject to conformational changes at high frequencies that are reflected by loss and reacquisition of transcriptional activity.

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