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Retransformation of a Revertant Cell Line With the Adenovirus E1 Oncogenes and Vanadate

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We have isolated a revertant cell line (G5) from an adenovirus transformed rat cell line (F4) which failed to express the integrated viral oncogenes. To determine whether the reversion mutation was acting in cis or trans the G5 cells were co-transfected with an E1 gene bearing expression plasmid and a neomycin phosphotransferase bearing plasmid. G418-resistant colonies were picked and shown to express the E1 proteins and to be tumorigenic. This re-transformation could be partially mimicked by treatment with vanadate, an inhibitor of phosphotyrosine phosphatases. These results show that the continued presence of the E1 proteins was required to maintain the transformed phenotype, and that the reversion mutation was a cis-acting event affecting directly the integrated E1 genes.

Key words: phosphatase phosphotyrosine, orthovanadate, viral oncogenes

Revertant cell lines isolated from rodent fibroblasts transformed by oncogenic viruses have been studied by several groups of investigators as an approach to define and ultimately resolve the mechanisms of malignant cell transformation [4,5,9,16]. We have isolated several revertants from an adenovirus-2 transformed embryonic rat cell line (F4) with the aforementioned objective in mind [7,11–13]. One of these revertants (G5) had the following properties: a) greatly reduced capacity for growth in soft agar, b) complete loss of tumorigenicity, c) loss of expression of the E1 viral transforming genes, d) no change in the restriction pattern and copy number of the integrated E1 genes. These properties suggested that either a viral or a cellular mutation was responsible for the loss of E1 gene expression and the consequent phenotypic reversion.

In this report we distinguish between these two alternatives by attempting to retransform the G5 cells genetically by DNA transfection with an E1 plasmid and phenotypically with vanadate, an inhibitor of phosphotyrosine phosphatases [3,14].

G5 cells were cotransfected with pLB206 and pZIPneoSVX. pLB206 carries the left 15.5% of the Ad2 genome and encodes both of the transforming genes contained in the E1 region [1]. pZIPneoSVX confers resistance to the antibiotic G418 in mammalian cells [2]. Control experiments were performed with phenotypically normal FR3T3 cells

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[10]. G418 resistant colonies and those showing a transformed morphology were scored. Table I shows that the G5 revertant was retransformed by reintroduction of new sequences of the E1 oncogene carried by pLB206. It is important to note that pZIP-neoSVX, which does not contain an oncogene, does not transform by itself. The integration of pLB206 resulted in the production of the E1a and E1b proteins as shown by immunoprecipitation (Table I, column 5) and reestablishment of the transformed (Table I, column 4), tumorigenic phenotype (Table I, column 6). This result argues against a cellular mutation which might have abrogated E1-responsiveness. The arrested expression of the E1 genes in the G5 revertant must therefore have been due to a cis-acting event, as opposed to a trans-acting one.

Vanadate has been reported capable of transforming normal cells, possibly via its demonstrated inhibitory effect on phosphotyrosine phosphatases [3]. Treatment with vanadate induced morphological transformation of the normal FR3T3 cells and the revertant G5 cells. A progressive change in the morphology of these cells was induced within 24 h, as measured by the reduction in cell diameter. This effect was fully reversible within 24 to 48 h by incubation in the absence of vanadate. The change in morphology was interpreted as indicating the appearance of a transformed phenotype as opposed to toxicity because the cells continued to divide normally in the presence of the drug. To pursue this further we also measured a more rigorous criteria of transformation, the capacity of vanadate to induce growth in agar. Vanadate dramatically increased the size and the number of colonies of FR3T3 in agar (Table II). This cell line normally gives a very low background of extremely minute colonies in agar, as opposed to the G5 cells which maintain a significant background level of growth. We noted a measurable increase in colony formation and a significant (2-fold) increase in colony size of vanadate-treated G5 cells, though this was not as dramatic as for the FR3T3 cells. The extent to which vanadate stimulates cell growth in soft agar is obviously limited by the degree to which the cell line already exhibits this property. Moreover, as reported before, this transformation parameter was induced by vanadate only to a limited extent and not equally in different cell lines [3]. The retransformability of the G5 cells with vanadate indicates that the phosphorylation of tyrosines on target proteins which are important to achieve transformation does not appear to be impaired by the reversion event. This reinforces the above conclusion that the continued presence of E1a gene products is

TABLE I. Retransformation of the G5 Revertant With E1 Genes

Cell line	Transfected DNA[a]	$G418^R$ colonies/ dishes in selection	Morphological transformation (%)	E1a, E1b proteins	Tumorigenicity in nude mice
Fr3T3	—	0/10	0	–	0/12
FR3T3	pZIPneoSVX	34/4	0	–	0/6
FR3T3	pLB206 + pZIPneoSVX	85/12	31	+	5/6[b]
G5	—	0/10	0	–	0/33[c]
G5	pZIPneoSVX	821/5	0	–	0/6
G5	pLB206 + pZIPneoSVX	1,176/7	10	+	7/9[b]

[a]Cotransfection of 20 μg of each plasmid by the calcium phosphate method.
[b]Two FR3T3-$G418^R$ clones and 3 G5-$G418^R$ clones were each injected at 3 sites in athymic nude mice (5×10^5 cells/site, subcutaneously) and the data indicates the total number of tumors recorded 50 days later.
[c]Reference 6.

TABLE II. Effect of Vanadate on the Growth of FR3T3 and G5 Cells*

Experiment	Cells	Na_3VO_4 (μM)	CFE + Na_3VO_4/ CFE control	Size of Na_3VO_4-treated colonies/size of control colonies
1	FR3T3	1	5.0	10
2	FR3T3	1	9.9	ND
	G5	1	1.5	ND
3	FR3T3	5	5.4	3
	G5	5	1.4	2

*FR3T3 and G5 cells were seeded in 0.33% agar with or without the indicated concentration of vanadate. The colony formation efficiency (CFE, %) was determined in duplicate for the treated cells and for the non-treated cells by counting 10–15 microscope fields per dish, 4 weeks after seeding. ND, not determined.

required to maintain the transformed phenotype, and that the reversion mutation was a cis-acting event impinging directly on the integrated E1 genes. Such a cis-acting event might involve changes in E1a gene promoter methylation. This possibility was examined in detail in several cell lines, including G5, by Weber et al. [15]. The E1a promoter region of F4 and G5 cells was equally digested by *Hpa*II, suggesting that this region was not methylated. The same conclusion was reached when the entire E1 coding region was examined [15]. Although these results rule out methylation of the E1 genes as the cause of transcriptional inactivation in G5 cells, they do not rule out the possibility of alterations in chromatin structure involving the integrated E1 genes and flanking sequences. Transfected thymidine kinase and globin genes were found to be coordinately regulated over a distance of 20 kilobases and dependent on the site of integration [8]. Phenotypic switching of the transfected polyoma middle T gene has been attributed to the presence of CpG islands in the vicinity of the integration site [6]. We tentatively conclude that reversion of the G5 cells resulting from transcriptional arrest of the E1 genes was not brought about by methylation within the E1 promoter or coding region but some other cis-acting event such as an alteration in chromatin structure over a region longer than the genes under study.

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