Functional *In Vitro* Assays for the Isolation of Cell Transformation Effector and Suppressor Genes

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Malignant transformation may be viewed as an imbalance between signals inducing cell growth and signals leading to growth inhibition, differentiation, or senescence. A basic understanding of how these counterbalancing forces interact to regulate normal cell growth is the prerequisite to comprehending the mechanisms of tumorigenesis. Identification and characterization of the gene products implicated in these regulatory pathways is the first step toward understanding the disease process. The studies outlined here provide the potential basis for isolating and molecularly characterizing transformation effector and suppressor genes, which must respectively function in the positive and negative regulation of normal cell growth. The general strategy used involves the isolation and molecular characterization of nontransformed variants (revertants) from populations of tumor cells. The selection of revertants is facilitated by the ability to separate normal from transformed cells by fluorescence-activated sorting. The basis for this separation is the differential retention of the fluorescent dye rhodamine 123 in the mitochondria of normal versus transformed cells. Using this approach, we have isolated revertants from a mutagenized population of v-fos-transformed Rat-1 fibroblasts. Characterization of these clones indicated that they had sustained causal mutations in transformation effector genes. The unmutated effector genes are being identified and molecularly cloned by isolating retransformed clones from revertant cell lines that have been transfected with DNA or cDNA from normal primary cells. The same selection protocol has also been used to isolate revertants from tumor cell lines that have been transfected with DNA or cDNA from primary cells. The putative tumor-suppressor genes present in these revertants are currently being analyzed.

**Transformation Effector Genes**

Dominant transforming genes or oncogenes were initially identified as the oncogenic elements of the acute transforming retroviruses. Within the context of a retrovirus, these oncogenes appear to be both necessary and sufficient for the initiation of tumorigenesis (1). It was the pivotal studies of Stehelin et al. (2) that first indicated that the retroviral oncogenes were not viral in origin, but rather were derived from cellular proto-oncogenes that had recombined with the viral genome during the course of infection. These findings provided concrete evidence for the notion that eukaryotic genomes harbored genes whose alteration and/or altered expression could give rise to the transformed phenotype. The subsequent search for oncogenes in human and animal tumors has resulted in the identification and molecular cloning of approximately 50 oncogenes and putative oncogenes (3). A great deal of information concerning the structure of these oncogenes, the proteins they encode, and the molecular mechanisms leading to proto-oncogene activation has been rapidly amassed. The functions of oncogenes and their corresponding proto-oncogenes in normal and transformed cells are also emerging. The different oncoproteins are localized in various compartments of the cell (nucleus, cytoplasm, cell membrane, or cytoskeleton) and have diverse functional characteristics including GTP-binding activity, tyrosine kinase activity, and function as growth factors, as soluble or membrane-associated receptors for growth factors or hormones, and as second messengers coupled to receptors (1,3–8).

Whereas much is known about the properties of the different oncogenes, less is known about the cellular components with which specific oncogenes interact and the cellular processes through which these oncogenes induce cell transformation. Theoretically, it should be possible to characterize cellular genes required for

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transformation by isolation and molecular characterization of revertants (nontransformed variants) from populations of cells transformed by specific oncogenes. Somatic mutations that could give rise to the revertant phenotype include those resulting in the inactivation of the oncogene, those resulting in the activation of dominant transformation suppressor genes, and those leading to the inactivation of effector genes involved in the cellular processes required for transformation by a given oncogene. Effector genes would include those genes whose expression is altered by the oncogene, genes that directly or indirectly affect the function of the oncoprotein, and genes comprising the complex molecular pathways that produce the pleiotropic phenotype of transformed cells.

A number of flat revertants have been isolated from populations of cells transformed by a variety of oncogenes. The majority of these, however, resulted from mutations affecting the expression of a functional transforming protein (9-19). A few cell lines arising from apparent mutations in cellular effector genes (20-24) or suppressor genes (25) have been isolated from rodent fibroblasts transformed by either v-ras, v-abl, v-mos, SV-40 T-antigen, or by adenovirus infection (26). These revertants were derived by treating mutagenized populations of transformed cells with various cytotoxic agents that are toxic to growing cells or cancer cells. Cultures were exposed to these agents under conditions where cells that had reacquired a nontransformed phenotype were contact inhibited and hence were less susceptible to these cytotoxic agents. These conditions therefore led to the preferential elimination of the transformed parental cells and after several cycles of growth followed by selective killing, permitted the isolation of morphologic revertants. In addition to being both inefficient and time consuming, this type of selection is based on differential growth parameters of normal versus transformed cells and hence may have precluded the isolation of certain classes of revertants. Moreover, the selection procedure itself may have induced epigenetic changes or changes in the number of chromosomes. Furthermore, if the cytotoxic agents used were themselves mutagenic, then their continuous presence during the selection period may generate a revertant phenotype resulting from multiple mutational events. While any of these mechanisms would result in the production of a revertant phenotype, the nature of these genetic or epigenetic changes may preclude their analysis by gene transfer experiments. It was therefore desirable to develop a rapid and efficient selection procedure that would permit the isolation of revertants resulting from a single mutational event.

The selection protocol developed is based on the prolonged retention of a fluorescent molecule within the mitochondria of many transformed cells relative to nontransformed cells (27-32). While the prolonged dye retention phenotype is neither essential nor sufficient for cell transformation, in many cells it appears to be tightly coupled to the mechanisms of transformation. Mutagenized populations of transformed cells can therefore be stained, destained overnight, and revertants selected by fluorescence-activated cell sorting. This procedure allows for isolation of revertants with unprecedented speed and efficiency and without exposure of the cells to additional selective pressures. It is therefore very unlikely that the selection procedure itself will contribute to induction of the revertant phenotype. Genetic changes in the resulting revertants should therefore reflect the specificity of the mutagen used.

Despite the fact that the prolonged dye retention phenotype is absolutely linked to cell transformation (33), our results have already validated the usefulness of this selection protocol in the isolation of revertant cell lines. Following random mutagenesis of v-fos-transformed fibroblasts, cells displaying a transient rhodamine-retention phenotype were selected by fluorescence-activated cell sorting. Characterization of the individual clones revealed that unlike the transformed parental cells, the revertants isolated with this protocol had a stable nontransformed morphology, were contact inhibited, failed to grow in soft agar, and were not tumorigenic when injected into syngeneic Fischer 344 rats or athymic nude mice. In addition, the revertant cells retained a functional transforming FBJ-MuSV that could be rescued by infection with a replication-competent Moloney murine leukemia virus. Immunoprecipitation experiments indicated that the levels of the p55 v-fos protein present in the revertants were comparable to the levels present in the transformed parental cells. Thus, the revertant phenotype was not the result of mutations affecting the activity or the expression of the oncogene present in the transforming FBJ-MuSV provirus. The revertants expressed a functional transforming v-fos gene, but were resistant to its transforming potential. More importantly, the revertants were also resistant to transformation by a variety of other oncogenes but could be transformed by the polyoma middle T-antigen as well as the neu and trk oncogenes. Taken together, these results indicated that the revertant cells had sustained mutations in one or more cellular genes that control transformation of Rat-1 fibroblasts by p55 v-fos and a variety of other oncogenes. These findings were consistent with the notion that several oncogenes may share common biochemical pathways leading to cell transformation.

**Strategy for Cloning v-fos Oncogene-Specific Transformation Effector Genes**

Somatic cell fusion experiments were used to establish the dominant or recessive nature of the revertant phenotype in each of the revertant clones. The results of these experiments indicated that the revertant phenotype was recessive in hybrids formed between each of the revertants and v-fos-transformed cells, ruling out the possibility that the revertant phenotype was induced by the activity of a dominant transformation suppressor gene. This interpretation was corroborated by
experiments in which the revertants were fused with nontransformed Rat-1 fibroblasts. The hybrid cell lines derived from these fusions reacquired a transformed phenotype, suggesting that the revertant phenotype resulted from the inactivation of transformation effector genes that were present in both normal and transformed fibroblasts. The dominance of the transformed phenotype in somatic cell hybrids indicated that it should be possible to identify the causal effector genes using DNA-mediated gene transfer experiments. The revertant clones have thus been transfected with genomic DNA or with cDNA libraries prepared in eukaryotic expression vectors with mRNA isolated from normal human and rodent fibroblasts.

Evidence from the analysis of these retransformants indicates that at least two of the revertant clones can be retransformed by vectors that constitutively express the c-jun proto-oncogene or the v-jun oncogene (J. Van Amsterdam, R. C. Sullivan, and H. Zarbl, in preparation). We have further shown that retransformants, generated by transfection of revertant clone EMS-1-19 with DNA from normal human cells, also harbor a c-jun-related gene. Although all these results are consistent with a causal mutation in the c-jun proto-oncogene, to date sequence analysis of about 80% of the nucleotides composing each of the c-jun alleles present in clone EMS-1-19 and Rat-1 cells have failed to detect mutations that might lead to an altered c-jun protein. An alternative interpretation of the data obtained is that the causal mutation in clone EMS-1-19 is actually in a c-jun-related sequence, and that the overexpression of c-jun or v-jun alleles is able to compensate for this mutation. Similarly, it is possible that the causal effector gene mutation is in a totally unrelated gene, and that jun oncogenes transform cells via a fos-independent mechanism. Additional experiments are required to distinguish between these possibilities.

The experimental results implicating c-jun as a putative v-fos transformation-specific effector gene led us to an independent experimental approach. The hypothesis that c-jun is a fos transformation effector gene predicts that c-jun is necessary for fos-induced cell transformation. Hence, inhibition of c-jun expression would be expected to inhibit v-fos-induced cell transformation. We have therefore generated vectors which are able to constitutively express anti-sense c-jun RNA. We have obtained preliminary data suggesting that it is indeed possible to suppress v-fos-induced cell transformation by expressing anti-sense c-jun mRNA. Analyses of the levels of c-jun mRNA and protein in these cells are being performed to test the validity of these observations.

**Tumor-Suppressor Genes**

It is generally accepted that oncogene-induced cell transformation represents a genetically dominant phenotype. However, studies performed over the last 20 years have clearly demonstrated that the cancer phenotype can also be recessive, giving rise to the hypothesis that there are genes present in normal cells that function as negative regulators of cell growth. These suppressor genes or anti-oncogenes differ from oncogenes in that their function must be lost before a cell can acquire a cancerous phenotype. Evidence for tumor suppression was first provided by studies demonstrating that the tumorigenic phenotype is usually suppressed in somatic cell hybrids between normal and transformed cell lines (32,33), with retransformed segregants arising only after loss of specific normal chromosomes. Repression of the cancer phenotype is also observed when malignant cells are introduced into certain embryonic environments (36–38). Evidence for suppressor genes is also derived from the analysis of the autosomal dominant patterns of inheritance of certain familial cancers. These analyses lead to the hypothesis that carriers were heterozygous for recessive mutations in suppressor genes. Cancers would then arise in individual cells that become homozygous for loss of suppressor gene function as a result of somatic mutations (39).

Over the past few years, this model has been confirmed by the identification of deletions in a defined region of chromosome 13 in human familial retinoblastoma by using cytogenetic observations and restriction-fragment-length polymorphism (RFLP) analyses with informative allele specific probes (40,41). Subsequent studies by several groups culminated in the molecular cloning of the retinoblastoma (Rb) suppressor gene (42,43) and in the demonstration that introduction of the cloned gene into retinoblastoma cell lines resulted in suppression of their tumorigenic phenotype (44). Although the loss of the Rb gene was initially identified with heritable forms of retinoblastoma and osteosarcoma, it is now clear that Rb gene mutation is also involved in sporadic retinoblastoma and in a variety of nonhereditary forms of cancer including small cell carcinoma of the lung (45,46), bladder carcinoma (47), and breast cancer (48,49).

Using approaches similar to those used to identify the Rb gene, deletions of specific chromosomal regions are being identified in an increasing number of human tumors (for recent reviews see Green (50), and Cavenee et al. (51)). A great deal of effort is thus being directed toward the molecular cloning of the putative tumor-suppressor genes associated with the specific chromosomal regions for which loss of heterozygosity is frequently seen in specific tumor types. The first approach being used to clone putative suppressor genes is the same as that which proved successful in cloning the Rb gene and involves chromosome walking from RFLP markers that flank the region containing the putative suppressor gene. This methodology has recently been applied to the analysis of alleles located on the short arm of chromosome 17 and on the long arm of chromosome 18, which are frequently deleted in human colorectal cancer (52–54). Results of this analysis indicated that the deleted regions on chromosome 17 include the p53 suppressor gene. Sequence analysis of the p53 alleles remaining in most colorectal cancer cells indi-
cated that they had sustained inactivating point mutations (53). Inactivating mutations in remaining p53 alleles were also seen in most brain, breast, and lung tumors that showed allelic deletions on chromosome 17p, and in some tumors without 17p allelic deletions (55). All of these results are consistent with the notion that inactivation of the p53 suppressor gene may be associated with several unrelated tumor types. Despite the success of the “brute force” approach described above, its application is limited to the analysis of those putative suppressor genes for which closely linked (within walking or jumping distance) RFLP markers are available.

An additional approach being employed to identify tumor-suppressor genes involves refinements of the somatic cell fusion technology, which first provided evidence for their existence. In this technique, normal cell lines in which single chromosomes are tagged with selectable marker genes are generated. Microcell fusion is then used to transfer single chromosomes into tumor cells, and cells that harbor the tagged chromosome are assayed for the presence of suppressor genes (51,56). The putative suppressor genes expressed from these chromosomes or chromosomal fragments could then be identified among the clones isolated by generating cDNA subtraction libraries between the parental tumor cell line and the revertant recipient cell (51,57) using functional suppression assays (see below). Alternatively, the suppressor gene residing in the exogenous chromosomes could be inactivated in the suppressed recipient cell by retroviral insertional mutagenesis and the viral sequences used to clone the inactivated suppressor gene sequences in the retransformed clones (53,59).

The methodologies outlined above are contingent on the ability of the suppressor gene to repress a selectable cancer phenotype in the recipient cell. However, recent results from several laboratories have provided evidence that loss of specific chromosomal regions containing putative suppressor genes may also play a role in malignant progression of a number of common human tumors (45,51–54,60,61). It can thus be speculated that it is the accumulated loss of heterozygosity (LOH) at several alleles that is responsible for induction of malignant cancers. The possibility therefore exists that individual suppressor genes associated with LOH for individual chromosomal regions may not be capable of inducing phenotypic reversion of tumor cells. Even though loss of each of these genes may contribute to tumor progression, it may not be detectable in suppression assays (cell fusion, microcell fusion, DNA transfection) dependent on a reduction in tumorigenicity of recipient cells. The ability to identify and clone such suppressor genes represents an even greater problem in those tumor cells where loss of gene function is not associated with a cytogenetically detectable LOH or by RFLP analysis. Such genes could only be detected by assays in which tumor cells at a specific stage of progression are shown to revert to a less aggressive histopathologic stage after introduction of a functional suppressor gene.

Reznikoff and her co-workers (62–64) have recently developed an experimental system that is uniquely suited to the identification of suppressor genes whose loss of function contributes to tumor induction and/or progression. These investigators produced a partially transformed, nonvirus-producing cell line by infecting primary human urethelial cells (HUC) with Simian virus 40 (SV40). This cell line (SV-HUC-1) was clonal, showed anchorage-independent growth in vitro, but failed to produce tumors in nude mice, even after more than 80 passages in vitro. While this cell line was found to be cytogenetically unstable, the continuous karyotypic rearrangements did not lead to tumorigenic conversion. The marker chromosomes formed were shown to be the result of balanced translocations, resulting in the production of a pseudodiploid cell line. The SV-HUC-1 cell line was then exposed to carcinogens in vitro, and tumorigenic progression monitored by injection of treated cells into athymic nude mice. The most important observation from these studies is that the resulting tumors collectively recapitulate the full spectrum of histopathology seen in human bladder cell carcinoma. Moreover, all cell lines isolated from tumors showed stable marker chromosomes and LOH for several chromosomal regions, with more malignant tumor types showing a greater accumulation of chromosomal changes.

The major advantage of this system is that all of the tumors generated are derived from the SV-HUC-1 cell line and hence should differ from one another by a limited number of genetic alterations. Suppressor genes whose inactivations are involved in tumor induction and progression could thus be identified using subtractive cDNA libraries prepared from tumors representing sequential histopathologic stages or by retroviral insertional mutagenesis as described above. Such studies would be much more difficult using tumors representing different histopathologic stages of progression that were not derived from the same individual.

An additional in vitro assay is currently being used to identify putative suppressor genes, particularly in those cases where the presumed loss of a suppressor gene is not associated with any particular cytogenetically visible change or with informative RFLP markers. This approach is contingent on the ability to detect suppressor gene activity in a functional assay. Thus, tumor cells are transfected with genomic DNA from normal cells (65) or with cDNA libraries from normal cells, prepared in eukaryotic expression vectors (66,67). Revertant cell lines, which have presumably acquired a functional suppressor gene, are then isolated and the putative suppressor genes characterized. Inherent in such an approach are two major drawbacks. The first potential problem arises if the gene that is able to suppress the transformed phenotype also suppresses cell growth. The inability to expand the resulting revertant cells would prohibit their characterization and analysis of the causal gene. It is therefore likely that functional
assays would detect only a subset of suppressor genes. In this regard the recent result demonstrating that the transformed phenotype of retinoblastoma cell lines can be suppressed by infection with retroviral constructs expressing the Rb gene (44) are reassuring and provide support for the feasibility of using such a functional approach. The second potential problem associated with using these gene transfer experiments is that the genes able to suppress cell transformation in the assay do so via a nonphysiological mechanism. It is probable that the constitutive overexpression of a gene could simply titrate away cellular factors required for cell transformation or simply gum up the biochemical machinery of the cell in a nonspecific way that will also inhibit cell transformation. Such transformation-reverting genes, while providing important information about the molecular biochemistry of cell transformation, could not be classified as true tumor suppressor genes. Nonetheless, it is anticipated that among the genes isolated by this approach will be some genes that are indeed tumor-suppressor genes. Indeed, the retinoblastoma gene could have been identified using a suppression assay (44).

Strategy for Cloning Human and Rodent Suppressor Genes

Using the functional approach described above, a number of genes able to suppress the transformation of fibroblasts by the K-ras oncogene have been recently cloned (65–67). A similar approach is also being used in our laboratory to isolate genes able to suppress v-fos-induced cell transformation and to isolate genes able to suppress the transformed phenotype of various human carcinoma cell lines.

Encouraged by the initial success in the isolation of revertants harboring effector gene mutations, we have applied the same techniques toward the isolation of revertants harboring transformation-suppressor genes. The approach taken is based on the assumption that transformation-suppressor genes normally function as regulators of cell growth and are thus expressed in nontransformed cells. We have, therefore, prepared cDNA from normal fibroblasts and cloned these cDNAs into eukaryotic expression vectors. DNA isolated from these cDNA libraries is subsequently cotransfected into transformed cell lines along with the pSV2 neo plasmid and transfectants selected with G418. Among the transfectants should be cells that are expressing a suppressor gene mRNA from a transfected cDNA expression plasmid (44,67). It should be possible to isolate the resulting revertant clones by fluorescence-activated cell sorting, given that the revertant phenotype includes loss of prolonged rhodamine retention. In a preliminary set of experiments (C. J. Kho and H. Zarbl, in preparation), we have been able to isolate several morphologic revertants from v-fos-transformed fibroblasts transfected with DNA from a human foreskin fibroblast cDNA expression library. At least one such revertant has been shown to contain a transfected cDNA expression plasmid, which is currently being characterized.

The prolonged rhodamine 123 retention phenotype is associated with most transformed cell lines of epithelial origin (29). We are applying our selection procedure to the isolation of suppressor genes that are able to revert the phenotype of tumor cell lines derived from the most common forms of human carcinomas. Cell lines derived from human carcinomas are therefore being transfected with cDNA expression libraries prepared with mRNA from either primary fibroblasts, or from cell lines derived from the same tissue as the corresponding tumors. The transfectants are then sorted on the basis of their dye retention phenotype in order to isolate revertants. Results of preliminary experiments are expected in the near future.

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