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Transformation Effector and Suppressor Genes

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Abstract Much has been learned about the molecular basis of cancer from the study of the dominantly acting viral and cellular oncogenes and their normal progenitors, the proto-oncogenes. More recent studies have resulted in the isolation and characterization of several genes prototypic of a second class of cancer genes. Whereas oncogenes act to promote the growth of cells, members of this latter class of genes act to inhibit cellular growth and are believed to contribute to the tumorigenic phenotype only when their activities are absent. This new class of cancer genes is referred to by a number of different names including; anti-oncogenes, recessive oncogenes, growth suppressor genes, tumor suppressor genes and emerogenes. Although only a few of these cancer genes have been identified, to date, it is likely that many additional genes of this class await identification. A third class of genes, necessary for the development of the cancer phenotype, is comprised of the transformation effector genes. These are normal cellular genes that encode proteins that cooperate with or activate oncogene functions and thereby induce the development of the neoplastic phenotype. The inactivation of transformation effector functions would therefore inhibit the ability of certain dominantly acting oncogenes to transform cells. The approaches outlined here describe functional assays for the isolation and molecular characterization of transformation effector and suppressor genes.

Key words: effector genes, suppressor genes, cancer, oncogenes, v-*fos*, rhodamine 123, non-tumorigenic revertants

Although cancer may be viewed as a prevalent cause of mortality of industrialized man, diseases with symptoms similar to that of cancer have been documented throughout the ages. Egyptian priests were the first to document their recognition of such disease symptoms as early as 3400 years ago [1]. Hippocrates, who lived in 500 B.C., is credited with introducing the name cancer, from the Greek word for crab, to describe the invasive growth of this ailment. In his attempts to understand the disease, Hippocrates was both limited by the technologies of the day and prejudiced by a limited understanding of human physiology. He believed that the body was composed of four humors—blood, phlegm, yellow bile, and black bile—and that a balance in the levels of these humors was required for good health. Cancer was believed to arise from an imbalance of the four humors that lead to an excess of black bile or melanchole [1]. Our present understanding of cancer indicates that his concept of an imbalance was fundamentally correct. Cancer arises not from an imbalance of the four humors, but rather from an imbalance between growth inhibitory and growth stimulatory signals processed by the cell.

Our understanding of altered growth stimulatory signals in cancer arose from the study of the dominantly acting retroviral oncogenes and their cellular counterparts the proto-oncogenes, which now number near 50 [2,3]. The products of the different oncogenes and proto-oncogenes have been found to function at virtually every step in the biochemical pathways that have been implicated in the control of cellular growth. Thus, oncogenes encode growth factors, growth factor receptors, and signal transducers which relay messages received at the cell surface to the cytoplasm and the nucleus. Other oncogenes encode nuclear transcription factors which control the expression of genes required for the induction of cell growth [3–6].

Although much has been learned about the function of several individual oncogenes, much less is known about biochemical networks through which they induce aberrant cell growth. These networks of biochemical pathways are composed both of growth stimulatory and growth inhibitory components. Genes whose products comprise and/or positively regulate the biochemical pathway of oncogene induced cell growth

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may be referred to as effector genes. Genes whose products negatively regulate cell growth may be referred to as suppressor genes. Transformation effector and suppressor genes serve to integrate growth stimulatory and growth inhibitory signals into precisely orchestrated alterations in gene expression that regulate cell growth and differentiation. Activation of effector genes or inactivation of suppressor genes will thus lead to the imbalances of these control mechanisms, which ultimately lead to initiation and/or progression of malignant cell growth. The identification and functional analysis of effector and suppressor genes is expected to contribute significantly towards our understanding of normal and abnormal cell growth [7–14]. Our laboratory has therefore focused on the development of strategies that permit the molecular cloning of these two classes of genes.

TRANSFORMATION EFFECTOR GENES

Effector genes may be defined as those genes whose products cooperate with oncogenes to induce cell transformation. Effector genes therefore would include 1) genes that directly or indirectly stimulate the function on an oncoprotein, 2) genes that must be stimulated by an oncoprotein or genes whose products must be activivated by an oncoprotein during the induction of the transformed phenotype, and 3) genes comprising the network of biochemical pathways that produce the pleiotropic phenotype of transformed cells. It is likely that many effector genes will also be proto-oncogenes that can themselves become oncogenes if activated by quantitative or qualitative mechanisms. An example of an effector gene is the PDGF receptor which is also the receptor for the *v-sis* oncoprotein [4]. In the absence of a functional receptor on the cell surface, the cell would not be responsive to the oncogenic potential of the *v-sis* gene. Other effector genes may include protein kinases, protein phosphatases, signal transducing G proteins, and transcription factors, as well as any other gene which serves to enhance or transduce a growth stimulatory signal into a growth response.

Our laboratory has focused on the development of functional assays that would permit the identification and molecular cloning of transformation effector genes. The feasibility of this approach is contingent on the ability to isolate non-transformed revertant cell lines from populations of transformed cells. If one assumes that transformation effector genes are essential for the induction of the transformed phenotype by a specific oncogene, then some of the revertants isolated may be the result of inactivating mutations sustained in essential effector genes. It should, therefore, be possible to use DNA-mediated gene transfer experiments to genetically complement and, hence, to clone the effector gene whose inactivation was responsible for blocking cell transformation.

The nature of the transformed cell gives it a selective growth advantage over most normal cells, not only in the unfortunate host, but also in many in vitro situations. This property of transformed cells has been used in DNA-mediated gene transfer experiments to identify foci of transformed cells within populations of normal cells and, hence, to molecularly clone dominantly acting oncogenes [15]. This same property of the transformed cells, however, makes it extremely difficult to isolate revertant cell lines. Revertants arising in a population of tumor cells would be expected to have normal cellular growth characteristics, including contact inhibition, and would therefore be selected against in culture. Revertants have been isolated using in vitro growth selection techniques analogous to chemotherapy used for cancer patients [16–22]. Populations of transformed cells (which may first be exposed to a mutagen) are treated with cytotoxic drugs that preferentially kill the faster growing, more aggressive transformed cells. Following such an enrichment process, revertants are selected on the basis of their normal morphology or growth characteristics. Using this approach, a few cell lines resulting from the apparent mutation of cellular effector genes or suppressor genes have been isolated from rodent fibroblasts transformed by either v-*ras,* v-*abl,* v-*mos,* SV40 T antigen, or by adenovirus infection [16–22]. As this type of selection procedure is based on differential growth parameters of normal versus transformed cells, the isolation of non-tumorigenic revertants retaining an aggressive in vitro growth phenotype may have been precluded. In addition, since many of the selective agents used are themselves mutagenic, the selection procedure itself may have generated the revertant phenotype by inducing additional genetic or epigenetic changes, a situation which would complicate the molecular analysis of such revertant cells by gene transfer experiments. We have

developed a novel selection procedure which does not require selective killing of tumor cells.

While studying the mitochondria of transformed cells using the mitochondria specific dye rhodamine 123, Chen and his coworkers [23,24] observed that in comparison to most normal cells, most transformed epithelial cell lines displayed a prolonged retention of this fluorescent dye. Our studies with rodent fibroblast cell lines have indicated that while the prolonged dye retention phenotype is not essential for cell transformation, it appeared to be tightly coupled to the mechanism of *v-fos* induced cell transformation [25]. Mutagenized populations of transformed cells can therefore be stained, destained overnight, and revertants displaying a transient dye-retention time can be selected by fluorescence-activated cell sorting. This procedure allows for isolation of revertants with unprecedented speed and efficiency, without exposure of the cells to additional selective pressures. It is very unlikely that the selection procedure itself will contribute to induction of the revertant phenotype, and genetic changes in the resulting revertants should reflect the specificity of the causal mutagenic agent.

Despite the fact that the prolonged dye retention phenotype is not absolutely linked to cell transformation in many cells, it appears to be tightly coupled to the mechanisms of transformation [23]. Furthermore, results have already validated the usefulness of this selection protocol in the isolation of revertant cell lines [25]. 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 by 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.

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 from 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 suppressing gene. This interpretation was corroborated by experiments in which the revertants were fused with nontransformed Rat-1 fibroblasts. Some of the hybrid cell lines derived from these fusions reacquired a transformed phenotype, suggesting that the revertant phenotype had 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 expression libraries constructed using mRNA isolated from normal human or normal rodent fibroblasts.

Although at least one putative effector gene has been identified using this approach (unpublished data), the ability of this gene to retransform the revertant is subject to alternative interpretations. First, it is possible that the putative effector gene is actually an activated proto-oncogene that is able to transform the cells via a v-*fos* oncogene independent mechanism. Alternatively, the putative effector may encode a gene that is functionally related to the causal effector gene and its overexpression in the revertant cell

compensates for the effector gene mutation. Studies are underway to distinguish among these alternative explanations.

The initial success of the selection procedure based on altered rhodamine 123 retention suggested that it should be possible to isolate revertants from any cell type displaying a prolonged retention time, including most human carcinoma cell lines. We have applied this selection protocol to human carcinoma cell lines and have succeeded in isolating a number of revertant cell lines (unpublished data). Characterization of these revertants should permit the identification of effector genes which may play a role in the genesis of human cancers.

A final approach being used in our laboratory to identify effector genes involves the isolation of genes that are differentially expressed between transformed cells and their corresponding revertants. We have found that α1(I) and α2(I) procollagen genes were expressed in Rat-1 fibroblasts, but not in v-*fos* transformants [26]. The genes were again expressed in the revertant clones, indicating that their regulation resulted from v-*fos*-induced transformation rather than from v-*fos* expression. In addition to the type 1 collagen genes, we have identified a number of other genes which show similar pattern of differential expression (unpublished data). These include several genes encoding nuclear transcriptional factors and mitochondrial genes. By studying the transcriptional regulatory regions of these and other differentially regulated genes, we hope to identify v-*fos*-transformation-specific transcriptional factors which function as effector genes in cell transformation.

TUMOR SUPPRESSOR GENES

Evidence for the existence of suppressor genes came from studies first performed over 20 years ago. These studies demonstrated that the tumorigenic phenotype was usually suppressed in somatic cell hybrids between normal and transformed cell lines, and occasionally in hybrids between different tumor cells [27]. Subsequent studies have shown that retransformed segregants arise only after loss of specific normal chromosomes, suggesting the presence of suppressor genes on these chromosomes [28]. Some experiments, such as those involving the hybridization of a human fibroblast to an EJ-bladder carcinoma cell containing an activated H-ras gene, indicated that the putative suppressor gene/genes could act dominantly over oncogenes [29]. More recent experiments have used microcell fusion technology to transfer specific chromosomes or chromosome fragments into tumor cells [30]. Experiments using this approach have not only corroborated the results from cell fusion experiments, but have also helped to define the physical map positions of the putative suppressor genes.

Evidence for the role of suppressor genes in cancer was also derived from the analysis of the autosomal dominant inheritance pattern of certain childhood cancers, including retinoblastoma and Wilms' tumor [31]. These analyses led to the hypothesis that the probands were heterozygous for recessive mutations in suppressor genes. Malignant transformation would then arise in individual cells that became homozygous for loss of suppressor gene function as a result of a somatic mutation. In recent years, a number of groups have undertaken the formidable task of isolating these tumor suppressor genes. The first such gene to be isolated was the Rb gene located at the retinoblastoma susceptibility locus [9–11]. Isolation of this gene was made possible by identification of its physical location within the human genome by linkage analysis. This was made possible after numerous cytogenetic observations indicated that a small percentage of retinoblastoma patients had deletions in a defined region of chromosome 13. Subsequent studies using restriction fragment length polymorphism (RFLP) probes were able to localize the region of interest [31] to the point where chromosome walking using these RFLP linkage probes made it possible to identify and clone the Rb gene locus. Verification that the Rb gene was, in fact, the suppressor gene whose inactivation is responsible for the induction of retinoblastomas has come from the ability of the cloned gene to suppress the tumorigenic phenotype when introduced into a number of tumor cell lines known to have defective Rb genes [32].

The identification of the Rb gene and its product, the 105 kDa nuclear phosphoprotein [33,34], has contributed substantially to our understanding of the function of growth suppressors and how alteration in their function may lead to cell transformation. For example, it was found that the Rb protein binds directly to the transforming protein of a number of DNA tumor viruses [33,34]. It has also been demonstrated that the binding is mediated by a conserved region of these viral proteins known to be essential for their transforming properties. The mechanism

of action of these transforming proteins is thought to involve the disruption of the growth suppressing activity of Rb and possibly other tumor suppressor proteins. Information on how the Rb protein itself may function to regulate cell growth has also accumulated. Although the Rb gene is expressed constitutively in all cells, its activity is modulated during the cell cycle by phosphorylation [35]. Rb is hypophosphorylated during G1, while it is hyperphosphorylated during S, G2, and M phases. As the SV40 virus oncoprotein binds to the hypophosphorylated form, it has been postulated that this form of the protein acts as a negative regulator of cell growth, possibly by repressing the expression of genes that would normally promote cell cycling [35]. This notion has been corroborated by recent studies showing that the levels of expression of the c-*myc* and c-*fos* proto-oncogenes, two genes with growth promoting activity, are negatively regulated by the Rb gene [36,37].

Using approaches similar to those used in the cloning of the Rb gene, a number of groups have identified additional candidates for tumor suppressor genes. Included are the Wilms' tumor susceptibility gene, WT-1 [12,13], the neurofibromatosis susceptibility gene, NF1 [38], and two genes frequently deleted in colorectal cancer, DCC (Deleted in Colorectal Cancer), and p53 [39]. Among these putative suppressor genes, only the p53 gene has been tested for the ability to suppress the transformed phenotype. Although the p53 gene was first characterized as an oncogene, it recently has been recognized as a tumor suppressor gene. Loss of heterozygosity at the p53 locus has been detected in a large number of different human tumors. Furthermore, gene transfer experiments have demonstrated that the wild type p53 gene has growth suppressing activity when expressed in a number of transformed cells [40]. Like the Rb protein, p53 is a nuclear phosphoprotein which can bind to polyoma large T antigen. In addition, oncogenic forms of p53 can bind to the cellular heat shock protein, Hsp70 [41]. The original classification of p53 as an oncogene came about due to its ability to immortalize cells and to complement ras in the transformation of primary rodent fibroblasts [41]. The confusion in the classification of p53 resulted from the cloning of a mutated gene from an established cell line which harbored mutant p53 genes [40]. Therefore, most of the initial experiments which investigators believed were being performed with a wild type clone of p53, were actually being performed with a mutant gene. Mutations in p53 are believed to contribute to its oncogenic potential. It has been suggested that the negative growth regulatory function of wild type p53 is disrupted by the formation of oligomeric protein complexes with mutant p53 and Hsp70 [40].

Although the approaches used to clone Rb and the other candidate tumor suppressor genes have been extremely successful, their application is limited to the analysis of those putative suppressor genes for which closely linked RFLP probes are presently available. In order to identify additional growth suppressor genes, a number of other possible approaches have been suggested. One alternative approach to cloning growth inhibitory genes involves refinements of somatic cell fusion technology [30,41,42]. In this technique, normal chromosomes are randomly tagged with selectable marker genes by gene transfer. Microcell fusion is then used to transfer single chromosomes into tumor cells, and then cells harboring the tagged chromosomes are assayed for the presence of suppressor genes. The putative genes expressed from these chromosomes could then be identified by generating cDNA subtraction libraries between the parental tumor cell lines and the revertant recipient cells. Alternatively, the suppressor gene could be inactivated in the suppressed recipient cells by retroviral insertional mutagenesis, and the viral sequences can then be used as a probe to clone suppressor gene sequences flanking the viral integration site.

Functional assays that make use of DNA-mediated gene transfer have also been used to identify a number of additional candidate tumor suppressor genes. Two of these genes, *myo*D (43) and $\alpha 5 \beta 1$ fibronectin receptor [44], which had been previously cloned, displayed suppressor activities when introduced into transformed cells. A third gene, K-*rev,* which encodes a protein with homologies to ras proteins, was also identified by gene transfer [44]. In these experiments rodent fibroblasts transformed by the K-*ras* oncogene were transfected with the DNA from a normal human fibroblast cDNA library prepared in a eucaryotic expression vector. The morphological revertants were identified after enrichment for cells which could survive or remain in culture under conditions which preferentially kill or remove transformed cells.

We have coupled the use of functional assays (expression cloning) for tumor suppressors genes with the procedure we already used to identify revertants of v-*fos* transformed rat fibroblasts [25]. In the present application, the protocol is used to isolate revertants which result from the expression of exogenous tumor suppressor genes. Thus, high molecular weight genomic DNA isolated from normal cells or DNA isolated from cDNA expression libraries constructed using mRNA isolated from normal cells are used as sources of exogenous genes. Revertant cells resulting from the activity of exogenously added suppressor genes are then separated from the transformed cells based on their differential rhodamine 123 retention properties. Although only transformed cells displaying a prolonged rhodamine retention time can be used in these studies, this remains a useful selection procedure, as many transformed cells have already been demonstrated to have prolonged retention times for the fluorescent dye [23–25].

There are several important considerations that must be weighed before embarking upon any attempt to clone suppressor genes by functional gene transfer assays (expression cloning). First, a variety of sources of normal donor DNA should be used, especially when using cDNA libraries, as suppressor gene expression may be tissue specific as seen in the case of Wilms' tumor suppressor gene [46]. Second, since revertants can only be isolated from among growing cells, a subclass of growth suppressor genes such as p53 may escape detection, although it remains theoretically possible to use vector specific primers to amplify cDNA inserts from nongrowing revertant cells using PCR. Nonetheless, experiments demonstrating that both the RB gene and the K-*rev* gene can suppress transformation without suppressing cell growth suggest that this approach remains a feasible one [32,45].

CONCLUSION

There is little doubt that identification of new tumor suppressor and effector genes will enhance our understanding of the molecular basis of cancer. This knowledge may lead to the rational design of therapies which would help to prevent the onset of disease or slow its progression. It has already been suggested that tumor suppressor genes may be used in gene therapy. The study of transformation effector genes may also lead to the rational design of cancer therapeutics. It may be possible to design inhibitors of transformation effector gene functions, thereby inhibiting oncogene functions. Many revertants isolated to date are resistant to retransformation by a number of different oncogenes ([25] and references therein). This observation leaves open the possibility that inhibition of a single effector gene function may be useful in the treatment of different cancers in which different oncogenes contributed to the development of the disease.

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