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Approaches to Proto-oncogene and Tumour Suppressor Gene Identification

Helen Patterson

INTRODUCTION

THE PAST decade has seen dramatic advances in our understanding of tumour development at the molecular level and it is now clear that the malignant phenotype arises as a result of the accumulation of genetic mutations in two classes of cellular genes, proto-oncogenes and tumour suppressor genes. Genetic alteration of proto-oncogenes causes their conversion into dominantly acting oncogenes and results in the altered expression of the oncogene or the production of an abnormal oncoprotein product. Although the demonstration in tumour cells of rearrangement, amplification or point mutation in a gene sequence is often taken as evidence that this sequence is acting as an oncogene, unequivocal proof can only be obtained by demonstrating transforming effects following expression of the cloned gene in, or introduction of the oncoprotein product, into the appropriate non-transformed cells. The considerable effort directed towards determining the function of these genes has clearly shown they are involved in the pathways by which growth factors promote normal cellular proliferation.

A substantial body of evidence now indicates that suppressor genes may also be involved in tumourigenesis. Although original accounts of these types of gene were restricted to rare inherited tumours, such as retinoblastoma and Wilms' tumour, it is now apparent that alterations of suppressor genes occur in most major classes of malignancy. In contrast to dominant oncogenes, where it is the presence of an abnormal gene product that is required for transformation, alteration of suppressor genes results in loss of genes that are involved in controlling cell growth and differentiation. In this case the complete removal of gene function requires inactivation of both copies of the gene. Proof that a gene has suppressor function can only be obtained from experiments in which tumour cells are reverted to a normal phenotype following introduction and expression of the normal cloned gene.

Following the original identification of oncogenes as the transforming sequences of acutely transforming retroviruses several different strategies have been used to detect activated genes that can contribute to tumour development. These methods, which will be reviewed below, have proven extremely successful and over 60 oncogenes and their corresponding proto-oncogenes have now been characterised. Although the identification of tumour suppressor genes has proven more difficult, a handful of genes, all of which have been implicated in human malignancy, have now been characterised and the strategies used in their isolation will also be considered.

PROTO-ONCOGENES

Retroviral oncogenes and integration sites

Two types of tumour-inducing retrovirus can be distinguished. One group (the acutely transforming retroviruses), which harbour viral oncogenes, is characterised by the ability to efficiently induce tumours in animals and to rapidly transform cells in culture. Over 20 retroviruses containing distinct oncogenic sequences have now been characterised and it is clear that in each case the viral oncogene (v-*onc*) was derived by transduction from a cellular gene, which is referred to as the cellular proto-oncogene (c-*onc*). Sequences originally identified as viral oncogenes include v-*myc*, v-H-*ras*, v-K-*ras*, v-*abl*, v-*src*, v-*erb*A and v-*erb*B [1] (Table 1). The v-*onc* and corresponding c-*onc* usually differ in their level of expression as v-*onc* sequences are under viral transcriptional control and may also be truncated or contain point mutations.

Chronically transforming retroviruses, so called because they induce tumours in animals with long latency, lack oncogenes but act via proviral integration in the host genome to disrupt cellular proto-oncogene sequences and their transcriptional control. Sequence analysis of viral integration sites in avian leukosis virus induced tumours has identified sequences previously cloned from acutely transforming retroviruses, e.g. c-*myc* [2] and c-*erb*B1 [3]. Sequences not previously cloned as viral oncogenes include *int*1 [4] and *int*2 [5], the common integration sites in

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Table 1. Human proto-oncogenes

Proto-oncogene	Involvement in human malignancy	Function
	Proto-oncogenes first identified as viral oncogenes of acutely transforming retroviruses	
c-*src*	—	Tyrosine kinase
c-*abl*	Rearranged by translocation in 80% CGL and 10% ALL	Tyrosine kinase
c-*myc*	Amplified: 20% small cell lung cancer. 10% breast cancer Rearranged in 100% Burkitt's lymphomas	Transcription factor
c-H-*ras* c-K-*ras*	Point mutations in codons 12, 13, 61 in several classes of human tumour, e.g. 70% pancreatic cancer, 40% colorectal cancer, 20% leukaemias (AML and ALL), also lung, melanoma and thyroid tumours	GTP-binding protein GTP-binding protein
c-*erb*B1	Amplified: 40% glial tumours, 20% squamous cell lung cancer.	Growth factor receptor
c-*erb*A	—	Thyroid hormone receptor
c-*jun*	—	Transcription factor
c-*fos*	—	Transcription factor
c-*fms*	Point mutations in 20% chronic myelomonocytic leukaemia and 20% M4 AML	GMCSF
c-*raf*	—	Serine/threonine kinase
	Proto-oncogenes detected by analysis of chronically transforming retrovirus integration sites	
int-1	—	Growth factor
int-2	—	Growth factor
pim-1	—	Growth factor
	Proto-oncogenes first identified by the NIH3T3 DNA transfection–transformation assay	
N-*ras*	Point mutations in several classes of human tumour	GTP-binding protein
met	Amplified in some gastric carcinoma cell lines	Tyrosine kinase
trk	—	Tyrosine kinase
dbl	Rearranged during transfection	—
ret	—	—
hst	Rearranged during transfection	Growth factor
mas	Rearranged during transfection	Receptor-like
	Proto-oncogenes identified by cloning	
bcl-1	Rearranged in 80% B-cell lymphomas	—
bcl-2	Rearranged in 80% follicular lymphomas	—
tcl-1	Rearranged in T-cell CLL	—
gli	Amplified in human glioma cell line	—
	Proto-oncogenes identified through sequence homology	
c-*erb*B2	Amplified: 25% breast carcinomas	Receptor-like
N-*myc*	Amplified: 50% late stage neuroblastomas, 20% small cell lung cancer and 20% retinoblastoma	Transcription factor
L-*myc*	Amplified: 15% small cell lung cancer	Transcription factor

Percentages are given only when a large number of tumours have been examined.

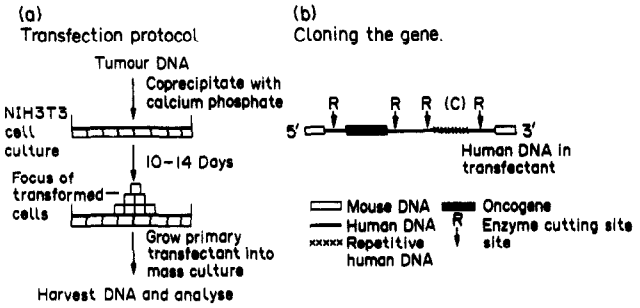

Fig. 1. (a) The stable uptake and incorporation of human DNA sequences into the mouse genome can be confirmed, without prior knowledge of the specific sequences transferred, by the use of probes homologous to human repeat sequences that are interspersed throughout the genome. If human sequences are confirmed, these same probes provide a starting point for cloning the gene. (b) Transfectant DNA is used to make a DNA library. The human specific repeat (XXXXX) probe will identify fragment (C) in this library. This fragment can be used to 'walk' (see Fig. 2) to the oncogene in a partially digested transfectant DNA library.

mouse mammary tumour virus induced breast tumours, and *pim*1 [6] the integration site in murine leukaemia virus induced T-cell lymphomas.

DNA transfection–transformation assay

Application of DNA to cells as a co-precipitate with calcium phosphate allows stable uptake and incorporation of DNA into the recipient cell genome [7]. Using this technique it has been shown that introduction of DNA from chemically transformed rodent cells [8], from human tumour cell lines and from primary human tumours [9, 10] into the NIH3T3 cell line can induce cellular transformation, identified by the appearance of foci of morphologically transformed cells on a background of non-transformed cells [8] (Fig. 1). These experiments demonstrated that many types of tumour contain activated cellular genes that can confer the transformed phenotype on the recipient NIH3T3 cells. In most cases the genes detected by this method are activated *ras* genes; K-*ras* and H-*ras* previously detected as viral oncogenes, and N-*ras* [11] first identified in this assay. These genes are invariably activated by point mutations at codons 12, 13 and 61. Several other genes including *neu* [12], *met* [13], *ret* [14], *raf* [15], *mas* [16], *hst* [17], *trk* [18], and *dbl* [19] (Table 1) have been identified by this route. The spectrum of genes cloned by transfection may only reflect the category of genes to which NIH3T3 cells are sensitive. In addition, genes spanning large regions of genomic DNA are unlikely to be transfected intact and for this reason will not be active in this assay.

Chromosomal translocations

Cytogenetic analyses have identified numerous specific chromosomal translocations in several classes of human malignancy. Many of these translocations have been shown to result in oncogene activation by rearrangement. The consistent translocation t[9; 22] found in chronic granulocytic leukaemia was one of the first reciprocal translocations to be characterised at the molecular level [20]. The search for candidate genes focused on the c-*abl* proto-oncogene mapped to chromosome 9 [21]. Southern blot analysis of hybrid cell lines, in which the der [22] (the Philadelphia chromosome) was the only human component, confirmed that c-*abl* sequences had been translocated to chromosome 22 [22]. DNA cloned in the vicinity of c-*abl* was found to contain sequences from both chromosomes 9 and 22 [23]

confirming that c-*abl* is disrupted by this translocation. The *bcr* gene on chromosome 22 is also involved. The translocation results in the production of a chimeric *bcr-abl* fusion protein which has elevated tyrosine kinase activity.

In Burkitt's lymphomas translocations involve breakpoints at 8q24, which becomes transposed to one of the immunoglobulin gene loci at 14q32, 2p13 or 22q11. Analysis of a candidate chromosome 8 proto-oncogene c-*myc* confirmed its role in these translocations [24]. The T cell receptor α, β and γ chain genes are also involved in translocations in leukaemias and lymphomas. For novel translocations involving immunoglobulin or T cell receptor genes it is possible to isolate the translocation partner by using these genes as probes to clone DNA spanning the breakpoint. Using this approach the proto-oncogenes *bcl*-1 [25], *bcl*-2 [26] and *tcl*-1 [27] have been cloned.

DNA amplification

Genes whose copy number is amplified in human tumours usually over-express the same gene. Hence amplification provides a mechanism by which oncogenes may be activated. Double minute chromosomes (DM) and homogeneously staining regions (HSR) are the cytogenetic hallmarks of gene amplification and are found in many types of human tumour. Several oncogenes cloned by other routes are known to be amplified in human tumours, e.g. c-*erb*B1 in glioblastomas [28] and N-*myc* in neuroblastomas [29], but one putative proto-oncogene *gli* was first cloned from amplified DNA in a human glioma cell line [30].

Structural homology

Genes identified as oncogenes by other methods have been used as probes to find related genes with sequence homology in DNA libraries. These genes are then examined for transforming activity or alteration in human tumours. *myc* gene probes have been used to isolate three related genes, N-*myc* amplified in neuroblastomas [29, 31], L-*myc* amplified in small cell lung cancer [32], and a third gene, R-*myc* [33]. c-*erb*B1 was used to isolate c-*erb*B2, which is amplified and over-expressed in 30% of breast cancers [34]. *Ras* related genes such as *rho* [35], *ral* [36], and R-*ras* [37] have been cloned, but these have not yet been implicated in human malignancy.

TUMOUR SUPPRESSOR GENES

Somatic cell hybrids and monochromosome transfer experiments

The earliest indication that malignant transformation might involve loss of normal gene function was provided by evidence of malignant suppression in somatic cell hybrids. The basic methodology is to fuse pairs of cells, one normal and one tumorigenic. Hybrid clones are selected and examined for their ability to form tumours in immunologically appropriate hosts, usually nude mice. Early cultures of hybrid clones were frequently non-tumorigenic, showing that malignancy had been suppressed, but on continued growth in culture they had a tendancy to re-express tumorigenicity. Subsequent cytogenetic analysis of non-tumorigenic hybrids and tumorigenic revertants showed that reversion correlated with loss of specific chromosomes [38, 39] which could be traced back to the normal fibroblast parent [40, 41]. The technique has been further refined through microcell transfer experiments in which single intact chromosomes are transferred to tumorigenic cells. Using this method tumorigenicity has been suppressed in HeLa cells by the introduction of human chromosome 11 [42], in a renal cell carcinoma cell line by chromosome 3p [43], and in melanoma cell lines by chromosome 6 [44]. The main problem with these studies is how to proceed from chromosomal location to molecular cloning. Although some genes cloned by other approaches may be active in this assay none have been directly isolated through this route.

Identification of tumour suppressor genes through the study of hereditary tumours

Retinoblastoma. On the basis of mathematical analysis of age-incidence data for familial and sporadic forms of retinoblastoma, Knudson proposed his two-hit hypothesis of retinoblastoma formation [45]. He suggested that, whereas sporadic tumours required two hits, hereditary tumours required only one hit, the first being inherited from the parent and carried in the germline. The original paper made no suggestion as to the nature of the two hits, but rare cytogenetic observations of constitutional deletions of chromosome 13q14 in hereditary retinoblastoma patients [46] provided evidence that the first or inherited mutation might involve loss of a region of chromosome 13. Loss of heterozygosity (L.O.H.) for polymorphic loci on chromosome 13 in tumour DNA from patients with familial retinoblastoma [47] has shown that frequently one copy of chromosome 13 has been lost. Furthermore, pedigree analysis [48] showed that the retained copy of chromosome 13 could always be traced back to the affected parent. These observations suggested that the second event might involve loss of a second allele at 13q14. To clone the gene Dryja *et al.* [49] constructed a chromosome 13 genomic library from which they cloned anonymous DNA segments. Those clones which mapped to 13q14 were used as probes in Southern blot analysis of germline and tumour DNA from retinoblastoma patients. One probe H3-8 detected homozygous deletions of DNA sequences at 13q14 in 3 of 37 retinoblastomas [50]. This probe was then used to commence a chromosome walk (Fig. 2) which led to the cloning of the retinoblastoma gene, RB1 [51, 52].

Wilms' tumour. Wilms' tumour has many parallels with retinoblastoma. Cytogenetic and chromosomal loss analyses of two predisposition syndromes WAGR and the Beckwith-Wiedemann syndrome have indentified predisposition loci at both 11p13 [53, 54] and 11p15 [55, 56]. Predisposition in some pedigrees maps to neither of these loci [57, 58] so a third may be involved. A candidate 11p13 gene has been cloned [59, 60]. Rose *et al.* [61] used irradiation reduced somatic cell hybrids, containing small fragments of chromosome 11 as their only

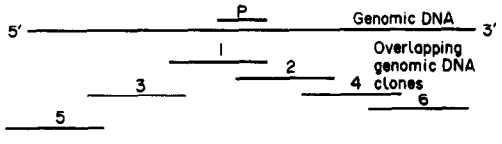

Fig. 2. A partial genomic library is constructed by the incomplete digestion of DNA with a restriction enzyme which has frequent cutting sites. This will generate a set of overlapping fragments which can be cloned into vectors and analysed. e.g. Probe P (which may define a locus near a gene of interest) can be used to screen the library (of overlapping genomic clones shown schematically in this diagram) and will identify clones (1) and (2). The most 5′ fragment of clone (1) and the most 3′ fragment of clone (2) can be used to rescreen the library to identify subsequent clones (3) and (4) which extend further in each direction. A further round of 'walking' will find clones (5) and (6). In this way large contiguous stretches of DNA may be cloned.

Table 2. Tumour suppressor gene loci defined by allele loss in loss of heterozygosity studies

Tumour	Chromosomal location	Corresponding tumour suppressor gene
Retinoblastoma	13q	RB1
Osteosarcoma	13q	RB1
Small cell lung cancer	3p, 13q, 17p	—, RB1, p53
Colorectal cancer	5q, 17p, 18q, 22	MCC, p53, DCC, —
Breast cancer	11p, 17p, 18q	—, p53, —
Wilms' tumour	11p	WT1 in some cases
Rhabdomyosarcoma	11p	—
Hepatocellular carcinoma	11p	—
Bladder cancer	11p	—
Renal cell carcinoma	3p	—
Meningioma	22	—
Acoustic neuroma	22	—
Phaeochromocytoma	1p	—
Medullary carcinoma of thyroid gland	1p	—

human component, to generate probes that were in turn used in pulsed field gel electrophoresis analyses to construct a complete physical map of the Wilms' tumour locus. Subsequent analysis of tumour cell lines identified a 345 kb region of common deletion which spanned a gene encoding a developmentally regulated [62], tissue specific transcription factor, WT1. The finding that the WT1 gene undergoes homozygous internal mutation in one case of Wilms' tumour [63] supports the claim that WT1 is the 11p13 Wilms' gene.

Neurofibromatosis type 1 (*NF*1). Linkage analysis of affected pedigrees [64] and the description of balanced translocations in two NF1 patients [65, 66] assigned the NF1 gene to 17q11.2. Subsequent mapping and cloning of these breakpoints resulted in the identification of a gene which harbours constitutional mutations in affected individuals [67, 68].

Loss of heterozygosity (L.O.H.) studies. The inherited mutation in familial retinoblastoma is usually a small deletion or point mutation. However, the loss of the second copy of the gene in tumour cells often involves loss of a large segment or all of the remaining normal chromosome. Such losses can be revealed as L.O.H. for adjacent polymorphic alleles in blot-hybridisation experiments of tumour DNA when compared to normal DNA. Thus, a consistent finding of L.O.H. for polymorphic markers at particular loci in tumour DNA may indicate that these loci harbour tumour suppressor genes. Using this type of analysis the sites of several putative tumour suppressor genes have been identified (Table 2) and in some cases the genes have been cloned. For example, allelic deletions of 18q occur in 70% of sporadic colorectal carcinomas and most frequently involve 18q21-qter [69]. Fearon *et al.* [70] identified a probe from this region which detected homozygously deleted DNA in one of 120 colorectal tumours, indicating that the probe was located extremely close to the suppressor gene. Following this breakthrough, the region pinpointed by this probe was cloned and found to contain a candidate suppressor gene, DCC, specifically mutated in colorectal carcinoma.

CONCLUSION

The concept that tumours arise in humans and laboratory animals from a single cell of origin, as a result of multiple, stepwise genetic mutations [71, 72] has its roots in a vast body of cytogenetic, experimental and epidemiological data [73–75]. The molecular analyses outlined above have been successful in identifying some of these mutations. However, only a few of the 60 proto-oncogenes so far identified have been directly implicated in human malignancy. An explanation for this may lie in discrepancies between *in vitro* models and animal models of tumorigenesis and tumorigenesis in humans. The difficulty in devising strong selection procedures for the negative phenotype has been one of the principle deterrents in identifying tumour suppressor genes [76] and explains why until recently their role in cancer development has been under represented. Progress has been made and the rate of this progress is accelerating. There is a great deal more to achieve, but the advent of molecular genetics has provided us with the tools to do the job.

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Identification and Interpretation of Epidermal Growth Factor and c-*erb*B-2 Overexpression

Barry A. Gusterson

Overexpression of normal cellular genes may be one mechanism by which malignant cells can acquire a selective growth advantage. The epidermal growth factor receptor and the c-*erb*B-2 protein are members of the *erb*B family and are good examples of genes that appear to act through this mechanism. Molecular and biochemical analyses of these two proteins also illustrate how studies of growth factors, growth factor receptors and oncogenic retroviruses may lead to new approaches to diagnosis and treatment. In particular, overexpression of these growth factor receptors has identified clinical subgroups that may respond differently to chemotherapy and provides the opportunity for antibody targeted therapy. Overexpression of these proteins can be identified using immunocytochemistry on both histological sections and fine-needle aspirates, thus enabling these parameters to be assessed preoperatively and to be monitored during therapy.
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INTRODUCTION

OVER 40 genes have now been described that appear to have a role in the malignant process. These genes often encode for proteins that are important components of the growth regulatory pathways in the normal cell [1]. In many cases these oncogenes are members of 'families' encoding proteins with similar structures and possibly similar functions [2].

Growth regulatory mechanisms usually involve the binding of a growth factor to a specific receptor on the cell surface, which then through an intracellular biochemical cascade leads to cell division. As different cells respond to different growth stimuli it is necessary for them to have cell type associated receptors. As the final response of the cell is similar, (i.e. cell division), it is not surprising that receptors have evolved with similar structures and often very close sequence homology in their amino acid composition. Where this homology is overt they are grouped into the same family often before their function is known. Thus newly identified proteins often have an assumed function based on our knowledge of the physiological role of other well characterised family members.

The epidermal growth factor receptor (EGFR) and the c-*erb*B-2 genes are examples of one of these families. They both encode transmembrane proteins that are putative growth factor receptors although they may have other functions in normal tissues [3–5]. Both of these receptors are overexpressed in certain tumour types. It is presumed that overexpression in some way increases the 'sensitivity' of the tumour cell to the normal levels of the growth factor that binds to the receptor. Alternatively increased levels of the receptor may produce a constitutively activated receptor cascade in the absence of the growth factor. It is proposed that both of these mechanisms would result in selectively triggering the proliferation of the tumour cell with a consequent selective growth advantage for the tumour population. It is not known *in vivo* which, if any, of these hypotheses is correct. However, antibodies to both the EGFR and the c-*erb*B-2 protein have been shown to inhibit tumour growth indicating that these molecules are important components of the growth response in these model systems. At

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