

# THE MOLECULAR GENETICS OF CELLULAR ONCOGENES

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## CONTENTS

INTRODUCTION .....	554
DEFINITIONS OF CELLULAR ONCOGENES .....	555
<i>c-onc's: The Proto-Oncogenic Progenitors of Retroviral Oncogenes</i> .....	555
<i>Identification of Cellular Oncogenes by Functional Tests for Dominant Mutations</i> .....	558
<i>Using Genetic Rearrangements to Identify Oncogenes</i> .....	562
<i>Proviral insertions can activate expression of cellular proto-oncogenes</i> .....	563
<i>Oncogenes at translocation breakpoints</i> .....	566
<i>Oncogenes in amplified DNA</i> .....	567
MOLECULAR PROFILES OF ACTIVATED ONCOGENES .....	570
<i>Base Substitutions in Mutant ras Alleles</i> .....	570
<i>Insertionally Mutated c-myc Loci</i> .....	574
<i>Locations of proviruses</i> .....	574
<i>Additional mutations in provirally mutated loci</i> .....	576
<i>Characteristics of Other Proviral Insertion Mutations in Proto-Oncogenes</i> .....	578
<i>Structural and Functional Properties of Translocation Breakpoints near Cellular</i> <i>Oncogenes</i> .....	581
<i>General features of c-myc; immunoglobulin gene translocations</i> .....	581
<i>Specific examples of c-myc; Ig translocations in PC and BL lines</i> .....	583
<i>The Ph<sup>1</sup> chromosome</i> .....	586
<i>Other translocations and rearrangements in B cell tumor lines</i> .....	587
<i>Structural Features of Amplified Oncogenes</i> .....	587
FROM <i>c-onc</i> TO <i>v-onc</i> : COLLUSION OF MUTATIONAL MECHANISMS .....	588
<i>The Favored Model for Transduction of Oncogenes</i> .....	590
<i>The Functional Significance of Differences Between c-onc's and v-onc's</i> .....	592
INACTIVATION OF INTEGRATED <i>v-onc</i> GENES: A SAMPLER OF MUTATIONAL MECHANISMS .....	593
NEW DIRECTIONS TOWARD A MOLECULAR DEFINITION OF CANCER .....	595
<i>Recessive Oncogenic Mutations</i> .....	595
<i>Mutations of Proto-Oncogenes in Genetically Malleable Hosts</i> .....	596
<i>Identifying Genes Whose Products Might Interact with Oncogene Proteins</i> .....	597
<i>Assessing Epigenetic Changes</i> .....	597
A CONCLUDING PERSPECTIVE .....	598

553

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## INTRODUCTION

Eventually the techniques of nucleic acid chemistry should allow us to itemize all the differences in nucleotide sequence and gene expression that distinguish a cancer cell from its normal counterpart, and perhaps at that point the steps involved in carcinogenesis will cease to be in doubt. (25)

Widespread interest in cellular oncogenes as potential substrates for the somatic mutations believed to underlie neoplastic change has brought students of cancer closer than could reasonably have been expected a mere four years ago to meeting the challenge laid down by Cairns (25). Efforts to define cancerous lesions at the nucleotide level have, moreover, been instructive on a broader front, providing a panoramic view heretofore unavailable of the kinds of mutations that afflict the somatic cells of higher eukaryotes.

My objectives in this review are to summarize the nature of the recently defined mutations of cellular oncogenes and to evaluate the hypothesis that they are giving us an accurate picture of both genetic change in somatic cells and the mutational basis of cancer. This evaluation must take into consideration the difficulties inherent in genetic attacks upon oncogenes: the poorly defined complexity of neoplastic phenotypes; the co-existence of multiple genetic changes in a single tumor cell; and the uncertain weight to be given to various lesions that may influence gene expression, alter the amino acid sequence of an oncogene product, or both. It is expressly not my intention to review the structure, origin, and biochemical functions of oncogenes; this has been accomplished in many other places in the recent past (16–18, 39, 253, 254). However, it seems appropriate to begin by describing the experimental strategies used to identify normal and mutant cellular oncogenes (Table 1), in part because the extent to which these strategies depart from classical genetics underlies current difficulties in correlating phenotypic changes in tumor cells with individual mutations.

**Table 1** Categories of cellular oncogenes based on mode of discovery

Proto-oncogenes	Active oncogenes
<i>c-onc</i> 's	<i>v-onc</i> 's <sup>a</sup>
Wild-type form of transforming genes	Transforming genes <sup>b</sup>
Wild-type form of rearranged genes	Rearranged genes <sup>c</sup>

<sup>a</sup> Cell-derived sequences to which oncogenic activity has been ascribed in retroviral genomes (see Table 2).

<sup>b</sup> Cellular genes competent to transform an appropriate recipient cell, e.g. NIH/3T3 cells (see Table 3).

<sup>c</sup> Cellular genes altered by insertion mutations (Table 4), chromosomal translocations (Table 5), amplification (Table 6), or other rearrangements.

## DEFINITIONS OF CELLULAR ONCOGENES

Much of the recent work in molecular oncology is motivated by faith in the proposal that a neoplastic cell develops from its normal progenitor as a consequence of changes (probably multiple) in some members of a restricted set of cellular genes. Such changes could be epigenetic, but the prevailing belief that cancer has its origins in mutations (24, 25) and the persuasive power of identified alterations in the structure of DNA (the subjects of this review) have focused attention primarily upon genetic events. The mutant genes are known as oncogenes, and corresponding wild-type alleles are called proto-oncogenes (or normal cellular oncogenes).

*c-onc's: The Proto-Oncogenic Progenitors of Retroviral Oncogenes*

Members of the largest group of proto-oncogenes have been identified experimentally by the homology of their nucleotide sequences to retroviral oncogenes, those regions of certain retroviral genomes believed to be responsible for swift induction of tumors and neoplastic transformation of cultured cells. The homologous cellular genes, known for convenience as *c-onc's* (35), are deemed proto-oncogenes because they have apparently served as targets for genetic transduction by retroviruses and thus are the normal cellular progenitors of viral oncogenes (*v-onc's*) (17, 18, 218) (Table 2).

Although the dividends of conferring the status of proto-oncogenes upon these cellular genes have been considerable, it must be acknowledged that the basis for doing so, the genetic definition of *v-onc's*, has not been uniformly rigorous. At one extreme, numerous conditional and non-conditional mutants of the *src* gene of Rous sarcoma virus (RSV) have convincingly demonstrated that *v-src* is necessary for both the initiation and maintenance of the transformed phenotype (125); other mutants of RSV, as well as manipulation of cloned *v-src* DNA, have established that the *v-src* gene is also sufficient for transformation (72, 122, 133a). At the other extreme, host-derived sequences present in the genomes of highly oncogenic viruses have sometimes been granted the provisional designation of *v-onc's* without supporting genetic evidence, even when the genome is also inhabited by another *v-onc* for which there is genetic confirmation of neo-plastic activity. Belief that such sequences have oncogenic potential of their own or the capacity to enhance the oncogenicity of accompanying oncogenes (e.g. by broadening the range of cellular targets for neoplastic effects) can be traced to at least three sources: (a) transduction of each cellular gene is a rare event for which there is likely to be some selective advantage for the virus (e.g. tumorigenic capacity); (b) in some cases of retroviruses bearing components derived from two independent cellu-

Table 2 Retroviral oncogenes (*v-onc*'s)<sup>a</sup>

Prototype virus(es) <sup>k</sup>	<i>v-onc</i>	Species of origin	Genetic basis			
			Conditional mutants <sup>b</sup>	Non-conditional mutants <sup>b</sup>	In vitro recombinant <sup>c</sup>	Multiple transduction <sup>d</sup>
RSV	src	chicken	+	+	+	
Y73/ESV	yes <sup>e</sup>	chicken	+			+
FuSV/PRCII	fps <sup>f</sup>	chicken	+		+	+
UR2	ros	chicken	+			
MC29	myc	chicken		+	+	+
MH2	myc mil <sup>g</sup>	chicken		(j)		+
AMV	myb	chicken	+			+
E26	myb ets	chicken				+
AEV	erbA erbB	chicken	+	+		+
SKV770	ski	chicken			+	
REV-T	rel	turkey		+	+	
Mo-MSV	mos	mouse	+	+	+	
Ab-MLV	abl	mouse		+	+	+
MSV-3611	raf <sup>g</sup>	mouse				+
BALB-MSV	Ha-ras <sup>h</sup>	mouse			+	+

Ha-MSV	Ha-ras <sup>h</sup>	rat		+	+	+
RaSV	Ra-ras <sup>h</sup>	rat				+
Ki-MSV	Ki-ras	rat	+		+	
FBJ-MSV	fos	mouse		+	+	+
FBR-MSV	fos	mouse				+
	fox					
ST-Fe SV, GA-FeSV	fes <sup>f</sup>	cat		+		+
SM-Fe SV	fms	cat				
GR-Fe SV	fgr <sup>e</sup>	cat				+
	actin <sup>i</sup>					
PI-FeSV, SSV	sis	cat, woolly monkey			+	+

<sup>a</sup> Further description and bibliography can be found in (16-18).

<sup>b</sup> Details and references can be found in (17, 18, 125, 126).

<sup>c</sup> + means that the *v-onc* sequence has been found to have transforming activity when moved to a new context by in vitro manipulation of cloned DNA (see 19).

<sup>d</sup> + means that the *onc* sequence has been encountered in two or more viruses that appear to have independently transduced the same *c-onc* from one or more host species (see 17, 18).

<sup>e</sup> *v-yes* and *v-fgr* appear to be derived from the same cognate *c-onc* (144a).

<sup>f</sup> *v-fps* and *v-fes* are derived from the same cognate *c-onc* (206).

<sup>g</sup> *v-mil* (also known as *v-mhr*) and *v-raf* are derived from the same cognate *c-onc* (102, 225).

<sup>h</sup> *Ha-ras* of BALB-MSV (also known as *v-bas*), *Ha-ras* of Ha-MSV, and *Ra-ras* of RaSV are all derived from the same cognate *c-onc*, called *c-Ha-ras* (6, 181).

<sup>i</sup> Part of the mammalian  $\beta$ -actin sequence is found in the genome of GR-FeSV (144a).

<sup>j</sup> A mutant of MH2 that appears not to express *v-mil* has normal or augmented oncogenic activity (161).

<sup>k</sup> Abbreviations: RSV, Rous sarcoma virus; Y73, Yamaguchi 73 sarcoma virus; ESV, Esh sarcoma virus; FuSV, Fujinami sarcoma virus; MC29, myelocytomatosis-29 virus; MH2, Mill Hill-2 virus; AMV, avian myeloblastosis virus; AEV, avian erythroblastosis virus; REV-T, reticulo-endotheliosis virus, strain T; Mo-MSV, Moloney murine sarcoma virus; Ab-MLV, Abelson murine leukemia virus; Ha-MSV, Harvey murine sarcoma virus; RaSV, Rasheed rat sarcoma virus; Ki-MSV, Kirsten murine sarcoma virus; ST-, GA-, SM-, GR-, PI-FeSV, Snyder-Theilen, Gardner-Arnstein, Susan McDonough, Gardner-Rasheed, Parodi-Irgens feline sarcoma viruses; SSV, simian sarcoma virus.

lar genes (e.g. *v-myc* and *v-mil* in MH2 virus), both components have been found singly in other transforming retroviruses (e.g. MC-29 and MSV-3611) (102, 225); and (c) in the case of avian erythroblastosis virus (AEV), mutants constructed in vitro suggest that one *v-onc* sequence (*v-erbA*) can potentiate the oncogenic action in the erythropoietic cell lineage of another, more critical sequence (*v-erbB*) (70, 203). For the majority of *v-onc*'s there is at least some supportive genetic evidence for oncogenic potential, although usually no direct evidence for a role in maintenance, since conditional mutations have only been isolated in a few cases (Table 2). More commonly the evidence is restricted to multiple isolations of the same oncogene in different highly oncogenic viruses and the retention of transforming activity when the viral oncogene is placed in a new context by the manipulation of cloned DNA.

For several years, the contention that the cellular progenitors of retroviral oncogenes might be the instruments of oncogenic change rested upon supposition. Even before the structural differences and similarities between *c-onc*'s and *v-onc*'s were recounted with the high resolution now possible, the two versions of each gene were recognized to be sufficiently alike to propose that partial recapitulation of events that produce active *v-onc*'s from *c-onc*'s might occur during tumorigenesis. Moreover, *c-onc*'s are well-conserved during metazoan evolution, commonly expressed in most or all tissues, and sometimes regulated in temporal or lineage-specific fashion (16), implying that they might play a central role in growth and development, processes that seem particularly likely to be altered during neoplasia. [The latter idea has received dramatic recent confirmation by the findings that *c-sis* appears to encode the platelet-derived growth factor (59, 251) and *c-erbB* the epidermal growth factor receptor (60).] However, new approaches were required to implicate *c-onc*'s directly in neoplasia.

### *Identification of Cellular Oncogenes by Functional Tests for Dominant Mutations*

The second means to identify cellular oncogenes perceives active oncogenes rather than proto-oncogenes. DNA transformation procedures, used for several years to demonstrate, for example, the oncogenic activity of nuclear DNA from cells containing integrated genomes of DNA and RNA tumor viruses, elicit neoplastic transformation when DNA from a wide variety of tumor cells is introduced into established lines of rodent fibroblasts, most commonly NIH/3T3 cells (39, 43, 207a, 253). Identified sources of active DNA by now include primary human tumors and cell lines; spontaneous and virus-, chemical-, and radiation-induced tumors of rodents and birds; and chemically-transformed cell lines (Table 3). The interpretation of such findings—an interpretation for which there is now considerable support (see below)—is that neoplastic transformation of the recipient cells is dependent upon the acquisition of a mutant allele.

The mutant allele is believed to act in a dominant manner in both recipient and donor cells, and its effect upon the recipient cells is alleged to simulate its biological activity in the donor cells. The presumption that a mutated gene is present in transformation-competent DNA was based initially upon the failure to induce the transformed phenotype in recipient cells with DNA similarly prepared from normal cells. However, the existence of a mutant gene in competent DNA requires more direct proof for several reasons: (a) sheared DNA from normal sources can transform cells at low frequency (43), presumably because under these circumstances strong promoters and proto-oncogenes from either donor or recipient cells can recombine to generate an efficiently expressed oncogene; (b) some wild-type *c-onc* proto-oncogenes (e.g. *c-mos*, *c-Ha-ras1*, and *c-fos*) can transform cells in culture when placed under the control of active transcriptional promoters (20, 31, 137, 159); and (c) rare acquisition of multiple copies of proto-oncogenes during transfection could presumably produce neoplastic change (akin to that proposed to occur as a result of gene amplification or transcriptional activation) without mutation in the donor cells.

Although the assay for oncogenes in NIH/3T3 cells has stimulated remarkable advances in our understanding of oncogenic mutations, it has come under criticism for an obvious reason: it offers a functional test for genetic lesions in a cultured mouse fibroblast that usually differs both in cell lineage and species from the tumor cells whose genes are under scrutiny. This potential weakness may well provide at least part of the explanation for two of the most striking characteristics of collective experience with the NIH/3T3 assay: the failure to observe transforming activity in DNA from 80–90% of spontaneous human tumors (39, 112, 174, 253) and the high proportion of active oncogenes that have proven to be mutant members of the *ras* gene family (see below). Recent attempts have been made to exploit other types of cell lines (193) or different assays for neoplastic effect (119; M. Wigler, personal communication) to seek additional oncogenes by DNA transfection. Although these alterations in methodology still have generally led to isolation of mutant *ras* genes, M. Wigler and colleagues (personal communication) have tentatively identified novel oncogenes in a human mammary carcinoma line, MCF-7, by treating NIH/3T3 cells with tumor cell DNA and a plasmid bearing a dominant genetic marker, then observing for tumor growth after inoculation of mice with pooled, co-transfected cells.

The NIH/3T3 cell assay also has been criticized on the grounds that the recipient cells, from an established line, are already abnormal. Inspired by requirements for two adeno- or polyoma virus genes to achieve full transformation of primary rodent embryo cells (62, 182), Land et al (114) and Ruley (192) have used embryo cells to show that two activated, molecularly cloned cellular oncogenes collaborate to produce a morphologically transformed cell with

**Table 3** Candidate oncogenes detected as transforming genes in the NIH/3T3 cell assay

Genes	Examples of cell types in which transforming activity is found <sup>a</sup>	References
<i>c-Ha-ras1</i>	Bladder carcinoma cell line (human)	55, 83, 112, 164, 197
	Urinary tract tumors (human)	70a
	Lung carcinoma cell line (human)	260
	DMBA and NMU-induced mammary carcinomas (rat)	220; see footnote c below
	DMBA/TPA-induced skin papillomas, benign and malignant (mice)	8, 9
	Melanoma cell line (human)	2
	Mammary carcinosarcoma line (human)	See footnote d below
	MC, BP, DEN and MNNG-transformed primary cells (guinea pig)	223
	Myeloid tumor cell line (mouse)	246a
	<i>c-Ki-ras2</i>	Lung carcinomas and cell lines (human)
Colon carcinomas and cell lines (human)		55, 174, 135
Pancreatic carcinoma cell line (human)		174
Gall bladder carcinoma cell line (human)		174
Rhabdomyosarcoma cell line (human)		174
Ovarian carcinoma, primary (human)		68
Acute lymphocytic leukemia line (human)		67
MC-induced fibrosarcomas and MC-transformed fibroblasts (mouse)		65, 165, 208
$\gamma$ -irradiation induced thymoma (mouse)		88
MC-induced thymic lymphoma and macrophage tumor lines (mouse)		246a
<i>N-ras</i>	BP-induced fibrosarcoma (mouse)	246a
	Neuroblastoma cell line (human)	210
<i>c-mos</i>	Burkitt lymphoma line (human)	77
	Fibrosarcoma and rhabdomyosarcoma cell lines (human)	89, 133
	Promyelocytic leukemia line (human)	143
	T cell leukemia line (human)	213a
	Melanoma cell lines (human)	2; see footnote e below
	Teratocarcinoma line (human, late passage)	228a
	Lung carcinoma line (human)	259a
	Acute and chronic myeloblastic leukemia (human)	67, 77
	Lung carcinoma cell line (mouse)	246a
	Carcinogen-induced thymoma (mouse)	88
<i>c-erbB</i> -related	Plasmacytoma cell lines (mouse)	36, 78, 184
<i>c-erbB</i> -related	ENU-related neuroblastoma cell lines (rat)	168, 208, see footnote f below

<i>Blym</i>	ALV-induced bursal lymphomas (chickens)	41, 42, 85
	Burkitt lymphoma lines (human)	56
<i>Tlym</i>	Intermediate T cell lymphoma lines (human)	117, 118
	MLV-induced T cell lymphomas (mouse)	117, 118
tx-1 <sup>b</sup>	Mammary carcinoma cell line (human)	116
	MMTV and DMBA-induced mammary carcinomas (mouse)	116
tx-2 <sup>b</sup>	PreB tumor cell lines (human)	117
	AbMLV-induced pre B tumor cell lines (mouse)	115
tx-3 <sup>b</sup>	Myeloma and plasmacytoma cell lines (human, mouse)	117
tx-4 <sup>b</sup>	Mature T cell lymphoma lines (human, mouse)	117
Unnamed	Pre B cell leukemia cell line (human)	160
Unnamed	MNNG-treated osteosarcoma cell line	38b

<sup>a</sup> Abbreviations: MC, methylcholanthrene; DMBA, dimethylbenzanthracene; TPA, tetradecanoyl-13-phorbol acetate; NMU, nitrosomethylurea; MNNG, N-methyl-N-nitro-N-nitroguanidine BP, benz(a)pyrene; DEN, diethylnitrosamine; ENU, ethylnitrosourea.

<sup>b</sup> Nomenclature suggested by M.A. Lane and G. Cooper (personal communication).

<sup>c</sup> M. Barbacid, personal communication.

<sup>d</sup> M. Kraus et al, personal communication.

<sup>e</sup> R. A. Padua, personal communication.

<sup>f</sup> R. Weinberg, personal communication.

tumorigenic potential. These results are provisionally interpreted to mean that one of the genes (an active form of *myc*, adenovirus E1A, or polyoma virus large T antigen gene) provides a nuclear function akin to that presumed to be active in established lines and required for prolonged growth, whereas the second gene (an active member of the *ras* gene family, adenovirus E1B, or polyoma middle T antigen gene) provides a cytoplasmic function responsible for the properties conventionally associated with the transformed phenotype (114). This sort of test appears to be particularly helpful for assessing the functional properties of putative oncogenes cloned from tumor cells (rather than for discovering new oncogenes) and for determining the contribution made by each member of a collaborating pair to the oncogenic phenotype in the recipient cell. However, it is uncertain whether two oncogenes are inevitably required (and hence whether they can rightly be called complementary genes): D. Spandidos & N. Wilkie (personal communication) have shown that a mutant *c-Ha-ras1* allele expressed under the control of a strong heterologous promoter does not require co-transfection with a *myc*-like gene for full transformation of primary rodent cells.

The likelihood that multiple mutations conspire to produce a full-fledged

cancer cell raises the disconcerting possibility that some of the mutations have phenotypic consequences that cannot be perceived in the absence of the others. One way to deal with this dilemma may be to exploit the traditional strategy of isolating phenotypic revertants of neoplastic cells (259). For example, partial phenotypic revertants of the human fibrosarcoma line HT1080 can be returned to a more advanced neoplastic state by transfection with the mutant *N-ras* gene formerly isolated from HT1080 cells with the NIH/3T3 cell assay (C. J. Marshall, personal communication). In a modest variation on this theme, non-tumorigenic cell lines established from human bladder cancers have been rendered tumorigenic by introduction of a mutant *c-Ha-ras1* gene from the human bladder cancer line, EJ (C. J. Marshall, personal communication). Again, however, phenotypic revertants or tumor cells with partially transformed phenotypes have not yet been used as target cells in DNA transfection studies designed to isolate new oncogenes from unfractionated tumor DNA.

The accumulated attempts to identify active oncogenes by transformation of cultured cells with tumor cell DNA have yielded a number of candidate oncogenes (Table 3), but the majority of positive results can be traced to one of three members of the *ras* gene family, a group of genes that encode closely related proteins generally about 21 kd in size. Two of these genes, known in the human genome as *c-Ha-ras1* and *c-Ki-ras2* (see footnotes to Tables 2 and 3), are members of the *c-onc* group; the third is called *N-ras*, a gene recognized as a member of the *ras* group by immunological cross-reactions of its product with antisera raised against products of other *ras* genes and, more definitively, by comparison of the deduced amino acid sequence of *N-ras* protein with corresponding sequences from other family members (210, 229).

Cooper and Lane and their colleagues have used the NIH/3T3 cell assay to implicate several genes outside the *ras* family as oncogenes; each oncogene is apparently specific for tumor cells within a cell lineage or at a certain stage in a cell lineage, as often deduced from the lists of restriction endonucleases that do and do not inactivate transformation competence (41, 42, 115–117) (Table 3). However, among these putative oncogenes only chicken and human *Blym* have been molecularly cloned and sequenced, and for these genes only transformation-competent alleles are available (56, 85). Hence, in no instance outside of the *ras* group has a mutation that renders a proto-oncogene competent to transform NIH/3T3 cells been proven by definition of the nucleotide sequence.

### *Using Genetic Rearrangements to Identify Oncogenes*

Some circumstantial evidence is very strong, as when you find a trout in the milk. (Thoreau)

Identification of genes that are structurally altered in tumors has proven to be an important if difficult means to register candidate oncogenes. Implicit in this endeavor is the assumption that a gene found to be altered in most cells in a

tumor is likely to be selected for and therefore contributory to the neoplastic phenotype. Clearly this is a hazardous simplification, particularly if, as suspected from some experiments (e.g. 32), cancerous cells have an altered mutation rate or if some extraneous mutations are highly favored to occur. To achieve a credible identification of a putative oncogene through a purely structural analysis, multiple sightings of the same gene in mutant form would seem to be a minimal requirement, some effect upon the expression or composition of its product is expected, and a detectable phenotypic consequence of introducing the rearranged allele into another cell is desirable, although infrequently available to date.

A structural approach to the discovery of oncogenes is, of course, further circumscribed by the complexity of the eukaryotic genome. Even the limited number of suspected targets for oncogenic mutations (e.g. the *c-onc* group of proto-oncogenes) presents too large a challenge to propose to seek mutations detectable only by nucleotide sequencing procedures. [There is a notable exception: base substitutions that alter restriction endonuclease recognition sites in certain *ras* genes occur with sufficient frequency that it is feasible to survey tumor DNAs for these mutations (c.f. 195).] Efforts to find mutant oncogenes, particularly novel ones, by a direct inspection of the genotype have been restricted to lesions that alter the mutant gene or its environs sufficiently to affect the mobility or abundance of its restriction fragments. Three kinds of genetic rearrangement have been found to affect *c-onc* proto-oncogenes, and each kind offers useful if laborious means to identify new oncogenes.

**PROVIRAL INSERTIONS CAN ACTIVATE EXPRESSION OF CELLULAR PROTO-ONCOGENES** Most retroviruses lack *v-onc* genes and fail to transform cultured cells but are nevertheless competent to induce tumors, generally after a protracted latent period (233). There is now broad support for the idea that many tumors that arise following infection by *v-onc*<sup>-</sup> retroviruses contain mutant cellular oncogenes activated by proviral insertions (243). It is generally held that such insertion mutations are primary events in tumorigenesis and that their effect is to stimulate expression of the target gene by the provision of a strong viral promoter or enhancer element within the viral long terminal repeat (LTR) (48a, 74, 94, 145a, 155, 166a, 167, 170a, 184).

Insertion mutations caused by the proviruses of horizontally transmitted retroviruses or of endogenous retrovirus-like elements have been found to afflict several cellular genes, some previously known as *c-onc*'s (*c-myc*, *c-erb B*, *c-myb*, *c-mos*, and *c-Ha-ras*) (Table 4). In most of these situations, the target gene for insertion mutation can be identified by examining restriction digests of tumor DNA for rearrangements using molecular probes for *c-onc* genes (74, 94, 147, 151a, 184, 217). Other candidate oncogenes (e.g. *int-1*, *int-2*, *pim*) have come to view as repeated targets for insertion mutation only by

applying methods akin to what has been called transposon tagging by students of mobile elements in *Drosophila* (14) (Table 4). To exploit this approach optimally requires that a tumor contain only a single copy of newly acquired proviral DNA. On the assumption that the single copy is acting as an insertional mutagen for an adjacent cellular gene, the viral DNA is molecularly cloned with some of the flanking DNA. Part of the retrieved cellular DNA is then used as a molecular probe to ask whether the same region of the host genome is occupied by insertions in other tumors (156). It has also been possible to isolate cellular sequences that appear to be targets for oncogenic insertion mutations from tumors bearing two or more proviruses (48a, 170, 236).

The conclusion that commonly interrupted cellular domains are sites of functionally important insertion mutations rests, in part, upon the assumption that retroviral proviruses integrate into the host genome without significant regional specificity. Although the available evidence argues that many sites in host chromosomes can accommodate proviral DNA (244a), it is premature to claim that integration occurs randomly. Other kinds of evidence are therefore used to sustain the view that the identification of candidate oncogenes by transposon tagging reflects the selective advantage conferred upon rare cells in which the insertion mutations occur, rather than integration at highly preferred sites. For example, the vast majority of the multiple MMTV proviruses in tumors with insertions in *int-1* or *int-2* are located outside these domains, and each tumor appears to carry an insertion in only one *int-1* or one *int-2* locus (156, 170). Evidence for an adjacent coding domain that is expressed at enhanced levels in the tumors and is evolutionarily conserved also fosters belief that the common insertion site harbors an oncogene (155).

In some instances—e.g. B cell leukemias induced by bovine leukemia virus, (86a, 105), T cell leukemias associated with T cell leukemia virus (204a), and nephroblastomas induced by myeloblastosis-associated virus (MAV) (157)—persistent efforts have failed to identify genetic targets for insertion mutation by probing for *c-onc* rearrangements or by transposon tagging, even though the tumors appear clonally derived. Such negative findings could imply that insertion mutations are not involved in these tumors, that several genetic targets are used, or that the insertions are remarkably far from the activated gene. Under certain circumstances, the existence of abundant transcripts from the mutated gene can assist in the identification and molecular cloning of the gene, e.g. when the transcripts contain viral sequences because the LTR serves as promoter for adjacent cellular sequences. For example, cloning of cDNA containing LTR sequences from a MAV-induced chicken nephroblastoma permitted the identification of the target gene for proviral insertion mutation as *c-Ha-ras*, despite the failure to perceive rearrangements by restriction mapping (D. Westaway, unpublished data).

Although heightened expression of an oncogene is believed to be the primary

**Table 4** Candidate oncogenes affected by insertion mutations

Gene	Insertion mutagen <sup>a</sup>	Tumor type	Species	Documented effect upon expression	References
<i>c-myc</i>	ALV, CSV, RPV	B cell lymphoma	chicken	+	71, 73, 94, 167
	CSV	B cell lymphoma	chicken		151a
	RPV	B cell lymphoma	chicken	+	see footnote c below
	MAV	B cell lymphoma	quail		see footnote d below
	Mo-MLV, MCF-MLV	T cell lymphoma	rat, mouse	+	43a, 217; see footnote e below
	FeLV	T cell lymphomas	cat		147
<i>c-erb B</i>	ALV	erythroblastosis	chicken	+	74
<i>c-Ha-ras</i>	MAV	nephroblastoma	chicken	+	see footnote d below
<i>c-mos</i>	IAP	plasmacytomas	mouse	+	36, 78, 184
<i>c-myb</i>	Mo-MLV	plasmacytoid lymphosarcomas	mouse		144; see footnote f below
<i>int-1</i>	MMTV	mammary carcinoma	mouse	+	156
<i>int-2</i>	MMTV	mammary carcinoma	mouse	+	170
<i>Mlvi-1</i>	Mo-MLV	T cell lymphoma	rat		236
<i>Mlvi-2</i>	Mo-MLV	T cell lymphoma	rat		235
<i>Mlvi-3</i>	Mo-MLV	T cell lymphoma	rat		see footnote g below
<i>RMO-int-1</i>	Mo-MLV	T cell lymphoma	rat		120
<i>pim-1</i>	MCF-MLV, MO-MLV	T cell lymphoma	mouse	+	48a
<i>c-raf</i>	MLV-LTR <sup>b</sup>	transformed fibroblast	mouse		140

<sup>a</sup> Abbreviations: ALV, avian leukosis virus; CSV, chicken syncytial virus; RPV, ring-necked pheasant virus; MAV, myeloblastosis-associated virus; Mo-MLV, Moloney murine leukemia virus; FeLV, feline leukemia virus; IAP, intracisternal A particle; MMTV, mouse mammary tumor virus; MCF-MLV, mink cell focus-forming murine leukemia virus.

<sup>b</sup> Cloned DNA containing an MLV LTR was used as an insertion mutagen via transfection.

<sup>c</sup> C. Simon, W. Hayward, personal communication.

<sup>d</sup> D. Westaway, unpublished data.

<sup>e</sup> G. Selton, A. Berns, Y. Li, N. Hopkins, P. O'Donnell, E. Fleissner, personal communication.

<sup>f</sup> G. Shen-Ong et al, personal communication.

<sup>g</sup> P. Tschlis, personal communication.

consequence of tumor-associated proviral insertion mutants, in some situations listed in Table 4, expression of the disrupted cellular domain has yet to be assessed. Furthermore, even in those instances (e.g. ALV insertion mutations of *c-myc* or MMTV mutations of *int-1*) in which tumor cells contain relatively high concentrations of RNA transcribed from the target gene, it is difficult to know whether the appropriate normal cell has been chosen for comparison. The insertions may also affect the efficiency of translation (e.g. by forming a shortened hybrid leader region in mRNA) or truncate the gene product (e.g. in ALV insertions within *c-erbB*). Lastly, it is now apparent that potentially significant alterations other than the insertion mutation may occur within affected loci; these will be described in greater detail below.

**ONCOGENES AT TRANSLOCATION BREAKPOINTS** It has been recognized for many years that chromosomal translocations are common in tumor cells and that certain combinations of chromosomes occur repeatedly in certain types of tumors (189–191, 261). Proto-oncogenes are sufficiently numerous and dispersed to inhabit virtually all chromosomes, so it is not credible to implicate a proto-oncogene in neoplasia merely because it is located somewhere within a partner in a frequent translocation event. Emphasis has instead been placed upon more stringent criteria: that a gene involved in the neoplastic process must be sufficiently close to the recombination site to have undergone detectable structural or functional alterations or must be within reach of the breakpoint as gauged, say, by restriction mapping with probes for adjacent sequences. Given the enormity of eukaryotic chromosomes, it is daunting to consider the routine identification of novel oncogenes by direct isolation of breakpoint regions. Sensibly, greater attention has been directed instead to the issue of whether proto-oncogenes known to be residents of translocated chromosomes might be positioned close to the breakpoints and whether their structure or expression is deranged as a consequence. To date, two known proto-oncogenes, *c-myc* and *c-abl*, have met the criteria that implicate them as oncogenic products of translocations (Table 5). A large number of B cell tumor cell lines of both human and murine origin harbor translocations that join the *c-myc* gene on one chromosome to one of the three immunoglobulin loci on another (107, 169, 191) (Table 5), in the manner originally predicted by Klein (106). The Philadelphia (Ph<sup>1</sup>) chromosome in chronic myelogenous leukemia (CML) links *c-abl* from one end of chromosome 9 to a restricted region of chromosome 22 (54, 87, 95). Novel putative oncogenes have been tentatively identified at chromosomal breakpoints in B cell tumors because they, like *c-myc*, are joined to immunoglobulin genes from the partner chromosome (Table 5).

The translocations of *c-myc* have been most closely studied and appear to

perturb expression of the oncogene by either transcriptional or translational mechanisms, with ancillary mutations within *c-myc* perhaps further augmenting the oncogenicity of the translocated allele; these issues will be addressed in a later section.

**ONCOGENES IN AMPLIFIED DNA** The initial sightings of amplified proto-oncogenes in human tumor cell lines grew out of attempts to explain unexpectedly high levels of expression of *c-onc* genes or to test the possibility that karyological markers of gene amplification in tumor cell lines—homogeneously staining regions (HSRs) or double minute chromosomes (DMs) (45)—might harbor *c-onc*'s (3, 37, 51, 199). Although a search for amplified oncogenes is inherently biased toward those proto-oncogenes already in hand, at least one novel oncogene (known as *N-myc*) has been discovered as an amplified gene in many neuroblastoma cell lines and tumors by virtue of limited homology with the second exon of *c-myc* (111, 198). Since methods for enriching genomic DNA for its amplified components are at hand—e.g. rate zonal sedimentation of DMs (80), gradient fractionation to prepare chromosomes enriched for markers bearing HSRs (104), isolation of middle repetitive DNA based upon reannealing kinetics (138), differential screening of cDNA libraries (111), and reannealing of restriction fragments within agarose gels (188)—candidate oncogenes may be advanced on the basis of their frequent amplification in certain types of tumors, even without any overt relationship to known proto-oncogenes. Other clues to amplified oncogenes have recently been exploited. The human tumor line A431, long known to express high levels of epidermal growth factor receptor (67a, 88a), was successfully tested for amplified numbers of *c-erbB* genes (124a, 136, 237a) when *c-erbB* was proposed to encode the receptor (60).

In all cases in which expression of amplified oncogenes has been examined, augmented levels of RNA have been encountered, although not always in direct proportion to the extent of amplification (201; F. Alt, personal communication). However, the effects of oncogene amplification may not represent a simple gene dosage phenomenon: preceding or consequent mutations within amplified genes, translocation to new chromosomal contexts, or co-amplification of adjacent genes may have additional influences (see below).

Amplifications of cellular oncogenes are proposed to influence tumor progression to advanced stages of malignancy. Two recent findings support this view: amplification of *N-myc* has been observed only in those neuroblastomas clinically assigned to Stages III or IV (22), and amplification of *c-myc* is regularly detected in those variants of small cell lung carcinoma cell lines with highly malignant growth potential (127).

**Table 5** Translocated cellular oncogenes

Tumor	Known or candidate oncogenes	Chromosome	Partner chromosome	Locus on partner chromosome	References
Mouse plasmacytoma cell lines	<i>c-myc</i>	15(D2/3)	12(F1)	IgH	1, 44, 46, 131, 205
Rat immunocytoxa cell lines	<i>c-myc</i>	7	6	?	224
Human Burkitt lymphoma cell lines	<i>c-myc</i>	8(q24)	14(q32) 2(p13) 22(q11)	IgH IgK Igλ	1, 47, 49, 50, 52, 98, 231
Human chronic myelogenous leukemia cell lines	<i>c-abl</i>	9(q34→pter)	22(q11)	<i>bcr</i>	87, 95
Human B cell leukemia and lymphoma	" <i>bcl-1</i> "(?) <sup>a</sup>	11(q13)	14(q32)	IgH	237a
Murine plasmacytoma	" <i>T<sub>K</sub>NS-1</i> "(?) <sup>a</sup>	10	6(C2)	C <sub>K</sub>	168
Murine plasmacytomias	" <i>pvt</i> "(?) <sup>a</sup>	15	6(C2)	C <sub>K</sub>	see footnote b below

<sup>a</sup>The oncogenic potential of these sequences is speculative.

<sup>b</sup>J. Adams, personal communication.

**Table 6** Amplification of cellular oncogenes

Oncogene	Tumor	Source	Degree of amplification	DM/HSR	Reference
<i>c-myc</i>	Promyelocytic leukemia cell line, HL60 and primary tumor <sup>a</sup>	Human	20×	a	51, 37, 153
<i>c-myc</i>	APUDoma cell line, COLO320	Human	40×	+/+	3
<i>c-myc</i>	Small cell lung carcinoma cell lines (variants)	Human	5-30×	?	127
<i>c-myc</i>	CSV-induced bursal lymphoma	Chicken	5-10×	?	151a
<i>N-myc</i>	Primary neuroblastomas (Stage III and IV) and neuroblastoma cell lines	Human	5-1000×	+/+	22, 111, 198, 201
<i>N-myc</i>	Retinoblastoma cell line, Y79, and primary tumors	Human	10-200×	+/+	111, 119a, 200
<i>N-myc</i>	Small cell lung carcinoma cell lines and tumor	Human	50×	+/?	See footnote c below
<i>c-abl</i>	Chronic myeloid leukemia cell line, K562	Human	5-10×	?	38, 95
<i>c-myb</i>	Colon carcinoma cell lines, COLO201/205	Human	10×	?	4
<i>c-myb</i>	Acute myeloid leukemia	Human	5-10×	?	167a
<i>c-erbB</i>	Epidermoid carcinoma cell line, A431	Human	30×	?	124a, 136, 237a
<i>c-Ki-ras2<sup>b</sup></i>	Primary carcinomas of lung, colon, bladder and rectum	Human	4-20×	?	See footnote d below
	Colon carcinoma cell line, SW480			?	135
<i>c-Ki-ras</i>	Adrenocortical carcinoma cell line, Y1	Mouse	30-60×	+/+	199
<i>N-ras</i>	Mammary carcinoma line, MCF7	Human	5-10×	?	see footnote e below

<sup>a</sup>DMs were observed in early passages of HL60 cells and an abnormal banding pattern in 8q was later observed by Nowell et al (153); both appear to coincide with *c-myc* amplification.

<sup>b</sup>In some of these tumors, rearrangements of *c-Ki-ras2*, with and without amplification, were also observed (M. Barbacid, personal communication).

<sup>c</sup>J. Minna, personal communication.

<sup>d</sup>M. Barbacid, personal communication.

<sup>e</sup>M. Wigler, personal communication.

## MOLECULAR PROFILES OF ACTIVATED ONCOGENES

Strategies for the identification of mutant cellular oncogenes are currently limited by their ability to detect only certain structural lesions (mainly rearrangements of various kinds) or lesions with certain functional consequences (transformation of cultured fibroblasts). Nevertheless, as summarized in the following sections, the observed genetic alterations seem to represent a diverse sampling of the possibilities for somatic mutation in vertebrate cells.

*Base Substitutions in Mutant ras Alleles*

Nucleotide substitution and concomitant amino acid change in members of the *ras* gene family is the genetic alteration that most commonly explains the transforming activity of tumor cell DNA (Tables 3, 7). The findings have particular force because it has been possible to use *in vitro* recombinants of wild type and mutant *ras* genes to validate the functional significance of the identified mutations (e.g. 28, 227, 260) and because changes affecting the same codons, 12 and 61, have been encountered in multiple mutant alleles of *c-Ha-ras1*, *c-Ki-ras2*, and *N-ras* (Table 7).

The reiterative pattern of these lesions raises two immediate questions: (a) what is the effect of amino acid changes at the observed positions on the behavior of *ras* gene products? (b) does the finding of repeated lesions at the same sites imply a hot spot for mutation or a selection for random mutations that confer transforming activity upon a proto-oncogene?

The prevailing hypothesis holds that mutations may occur at various sites in *ras* genes (and presumably throughout the genome), but that strong selective pressures favor the outgrowth of cells that undergo changes in a restricted number of sites within coding regions. It has been conjectured, for example, that replacement of the glycine residue normally encoded by the twelfth codon of *c-Ha-ras1* by amino acids with side chains will significantly alter the conformation of p21<sup>*c-Ha-ras*</sup> or the nucleotide binding site predicted to reside in this domain (171, 196, 258). Support for the functional importance of positions 12, 59, and 61 in p21<sup>*c-Ha-ras*</sup> and for the likelihood that random mutations at these sites are selectable has come from three genres of *in vitro* mutagenesis experiments. First, A. Levinson & W. Colby (personal communication) have used synthetic oligonucleotide primers to generate mutants of *c-Ha-ras1* encoding 18 of the possible amino acids other than glycine at the twelfth position. All of these, save pro-12, are capable of transforming NIH/3T3 cells, although the phenotypes vary from highly transformed to relatively subtle ones. Second, Fasano et al (67b) have used sodium bisulfite to produce C to T transitions at many positions in *c-Ha-ras1* and then have tested cloned mutants for their ability to transform NIH/3T3 cells. Oncogenic mutations occurred only at a restricted number of sites, including codons 12, 13, 59, 61, and 63. Three of

these positions conform precisely to the positions of mutations encountered in spontaneous tumors and in viral *ras* oncogenes (Table 7), and all are close to such sites. The change observed at position 59, ala  $\rightarrow$  thr, is particularly interesting because it is one of the three differences in amino acid sequence between c-Ha-*ras*1 and v-Ha-*ras*, implying that the viral oncogene has acquired two alterations independently sufficient to induce cellular transformation. This prediction has been confirmed by showing that a virus bearing a v-Ha-*ras* gene with gly-12 and thr-59 is as oncogenic as wild-type Ha-MSV (P. Tambourin, D. Lowy, personal communication). Third, K. C. Vousden and C. Marshall (personal communication) have mutated c-Ha-*ras*1 in vitro with a diol epoxide and found that five of twenty-one resultant transforming genes have restriction site changes in the eleventh or twelfth codon.

The identification of single nucleotide substitutions, both transitions and transversions, that account for the activation of *ras* proto-oncogenes in spontaneous human tumors (Table 7) is consonant with the widely disseminated notion that environmental mutagens serve as carcinogens (5). Further support for this idea has emerged from the isolation and structural analysis of mutant *ras* alleles from tumors induced experimentally in rodents with a variety of chemical carcinogens (Tables 3 and 7). For example, with striking reproducibility, both dimethylbenzanthracene (DMBA) and nitrosomethylurea (NMU) induce rat mammary tumors bearing activated c-Ha-*ras*1 alleles, the lesions in all examined instances being G to A transitions in the twelfth exon (22; M. Barbacid, personal communication). In the classical two-stage model for induction of skin carcinomas in the mouse with an initiator (e.g. DMBA) and tumor promoter, Balmain & Pragnell (8) have shown that the c-Ha-*ras* gene is activated, even at a relatively early stage (benign papillomas) (9). Similarly, mouse cells transformed in culture with methylcholanthrene (MCA) and MCA-induced murine fibrosarcomas carry activated c-Ki-*ras* genes (65, 165, 206a, 246a).

Some central issues and assumptions about *ras* point mutations remain uncertain, if not controversial:

1. Are *ras* mutations dominant? Although most evidence favors the idea that these mutations act in a simple dominant fashion, it is not uncommon to encounter results that undermine confidence in that assumption. Some tumor lines appear to carry only the mutant allele and hence are homo- or hemizygous for it, as judged by analysis of restriction sites that overlap the altered codon (195); in one instance, the human cell line SW480, the same twelfth codon mutation in c-Ki-*ras*2 is present in two alleles distinguished by an apparent polymorphism in a non-coding region (29). Furthermore, mutant alleles are sometimes amplified [e.g. a four- to eight-fold increase in c-Ki-*ras*2 in SW480 cells (135)], duplicated by tetraploidy [e.g. in the EJ bladder carcinoma line (U. Franke, personal communication)], or expressed more frequently than the

Table 7 Mutations in transforming *ras* genes

Source of allele	<i>ras</i> allele	Codons			References
		12	59	61	
Normal	Human c-Ha- <i>ras</i> 1	<u>GGC</u> gly	<u>GCC</u> ala	<u>CAG</u> gln	28
Normal	Rat c-Ha- <i>ras</i> 1	<u>GGA</u> gly	?	?	222
Ha-MSV	v-Ha- <i>ras</i>	<u>A</u> GA arg	ACA thr	CAA gln	55a
RaSV	v-Ra- <i>ras</i> <sup>a</sup>	<u>A</u> GA arg	GCA ala	CAA gln	181
Bladder Ca lines	Human EJ/T24-Ha- <i>ras</i> 1	<u>G</u> T <u>C</u> val	<u>GCC</u> ala	<u>CAG</u> gln	227, 196, 186, 28, 230
Lung Ca line	Human HS242-Ha- <i>ras</i> 1	<u>GGC</u> gly	<u>GCC</u> ala	<u>C</u> T <u>G</u> leu	260
Mammary Ca	Rat NMU-Ha- <i>ras</i> 1	<u>G</u> A <u>A</u> glu	?	?	222
Normal	Human c-Ki- <i>ras</i> 2	<u>GGT</u> gly	<u>GCA</u> ala	<u>CAA</u> gln	135a
Ki-MSV	v-Ki- <i>ras</i>	<u>A</u> GT ser	<u>A</u> CA thr	CAA gln	237
Lung Ca line	Human Calu-Ki- <i>ras</i> 2	<u>T</u> GT cys	GCA ala	CAA gln	209, 29
Colon Ca line	Human SW480-Ki- <i>ras</i> 2	<u>G</u> T <u>T</u> val	GCA ala	CAA gln	29

Lung Ca	Human LL-10-Ki- <i>ras</i> 2	$\begin{array}{ c } \hline \text{C} \text{GT} \\ \hline \text{arg} \\ \hline \end{array}$	?	?	195
Lung Ca line	Human A2182-Ki- <i>ras</i> 2	$\begin{array}{ c } \hline \text{C} \text{GT} \\ \hline \text{arg} \\ \hline \end{array}$	?	?	195
Bladder Ca line	Human A1698-Ki- <i>ras</i> 2	$\begin{array}{ c } \hline \text{C} \text{GT} \\ \hline \text{arg} \\ \hline \end{array}$	?	?	195
Lung Ca line	Human PR371-Ki- <i>ras</i> 2	$\begin{array}{ c } \hline \text{T} \text{GT} \\ \hline \text{lys} \\ \hline \end{array}$	?	?	145
Lung Ca line	Human PR310-Ki- <i>ras</i> 2	$\frac{\text{GGT}}{\text{gly}}$	$\frac{\text{GCA}}{\text{ala}}$	$\frac{\text{CA} \text{T}}{\text{his}}$	see footnote b below
Normal	Human N- <i>ras</i>	$\frac{\text{GGT}}{\text{gly}}$	$\frac{\text{GCT}}{\text{ala}}$	$\frac{\text{CAA}}{\text{gln}}$	229
Neuroblastoma line	Human SK-N- <i>ras</i>	$\frac{\text{GGT}}{\text{gly}}$	$\frac{\text{GCT}}{\text{ala}}$	$\frac{\text{A} \text{AA}}{\text{lys}}$	229
Teratocarcinoma line, late passage	Human PA1-N- <i>ras</i>	$\frac{\text{G} \text{A} \text{T}}{\text{asp}}$	?	?	228a
Fibrosarcoma line	Human HT1080-N- <i>ras</i>	?	?	$\frac{\text{A} \text{AA}}{\text{lys}}$	see footnote c below
Malignant melanoma line	Human Mel N- <i>ras</i>	$\frac{\text{GGT}}{\text{gly}}$	$\frac{\text{GCT}}{\text{ala}}$	$\frac{\text{A} \text{AA}}{\text{lys}}$	see footnote d below
Lung carcinoma line	Human SW1271 N- <i>ras</i>	$\frac{\text{GGT}}{\text{gly}}$	$\frac{\text{GCT}}{\text{ala}}$	$\frac{\text{C} \text{G} \text{A}}{\text{arg}}$	259a

<sup>a</sup> Amino acid positions aligned with those of other *ras* proteins.

<sup>b</sup> M. Perucho, personal communication.

<sup>c</sup> A. Hall, C. Marshall, personal communication.

<sup>d</sup> R. A. Padua, personal communication.

normal allele [e.g. in the CaLu tumor line (29)]. And when introduced into the NIH/3T3 cell containing normal *ras* alleles, it is usual to observe several copies of the mutant gene. On the other hand, it has been possible to demonstrate heterozygosity at the mutant locus in some instances [e.g. a primary human lung carcinoma (195)]. On balance, it seems likely that the *ras* mutations are dominant but dose-dependent, with consequent selection for cells in which the mutant allele is augmented by chromosomal duplication, gene amplification, gene conversion, or preferential expression. It is still uncertain how the findings with *ras* alleles are to be reconciled with evidence that at least some aspects of the oncogenic genotype behave as recessive properties in somatic cell hybrids (e.g. 214).

2. When are *ras* mutations acquired? Although it is generally held that *ras* mutations arise somatically during oncogenesis, only in rare instances has this hypothesis been directly validated: e.g. in chemically induced tumors in inbred mouse strains (222) and in a patient whose lung carcinoma contained a mutation that created a *SacI* site not present in multiple samples of normal tissue (195). Greater debate, however, surrounds the question of whether *ras* mutations are early or late events in tumor cell development, a question that can be extended to consider the possibility that the mutations are merely products of the exaggerated mutation rates observed in tumor cells (32). There are now numerous instances of mutant *ras* alleles in primary tumors of man and animals (Tables 3 and 7), countering earlier proposals that such mutations occurred only in cultured cells. Moreover, mutations in c-Ha-*ras* appear to be early events in chemically induced skin tumors, as is evident by transformation assays when the tumors are still benign by traditional criteria (9). Based on studies of human melanoma (2) and teratocarcinoma (228) lines, however, it has been argued that *ras* mutations may be late events in some settings; most tellingly, only one of five lines derived from a single melanoma patient exhibited a mutant gene, implying either that the mutation arose in culture or that the tumor was heterogeneous with respect to the lesion. At present, it appears that *ras* mutations are somatic events that can occur at various stages of the neoplastic process, but better functional tests will be required to establish the contribution the lesions make to the cell types in which they are encountered.

### *Insertionally Mutated c-myc Loci*

**LOCATIONS OF PROVIRUSES** A large number of insertion mutations caused by ALV, CSV, and MLV proviral DNA introduced into *c-myc* loci have now been analyzed structurally in DNA from avian bursal and rodent T cell lymphomas. The *c-myc* genes of chickens, mice, and man appear to consist of three exons (239), the first of which is non-coding and presumed to have a regulatory function (e.g. 119). Mapping with restriction endonucleases indicates that most

of the insertions in bursal lymphomas reside within the first intron of *c-myc* between the non-coding 5' exon and the exon that contains the apparent initiation site for translation of *c-myc*; furthermore, most of the proviral mutagens are oriented to allow their LTRs to serve as promoters for *c-myc* (71, 94, 167; R. Swift, H. Robinson, personal communication). Since there is little reason to believe that retroviral integration occurs preferentially at certain sites in host genomes or with preferred orientation, it seems likely that the commonly encountered configurations of viral DNA in *c-myc* loci imply a high probability of efficient expression of *c-myc*. Proviruses within the first intron appear to be clustered in a fashion that conforms to the idea that insertions at some sites are selectively advantageous over insertions at other sites (207; R. Swift, H. J. Kung, personal communication). Nucleotide sequencing of a few of the host-proviral junctions (146, 256; R. Swift, personal communication) shows that the insertions are at different positions within the intron without apparent homologies between insertion sites or between host and viral sequences at the recombination sites, findings consistent with studies of host-viral junctions from proviruses not known to affect the behavior of adjacent cellular genes (244a). The clustering of proviruses within the *c-myc* intron is particularly likely to be affected by the presence of nearby sequences that can serve as splice donor sites to join with the splice acceptor site at the 5' end of the second exon of *c-myc*. Insertions have been found within the splice donor site from the first exon of *c-myc* (146; C. Nottenburg; unpublished data), just upstream of a cryptic splice donor site that was also used during the transduction of *c-myc* to generate the *v-myc* gene in MC-29 virus (207, 252, 256), and on the 5' side of another intron sequence that conforms to the splice donor consensus (146).

Other arrangements have been encountered within insertionally activated *c-myc* loci in bursal lymphomas: proviral DNA in the first intron (or further upstream) in the opposite transcriptional orientation [(167); M. Linial, cited in (90)], on the 3' side of the coding domain in the same transcriptional orientation (167); and on the 5' side of the first exon in the same orientation (162; H. Robinson, personal communication). In the former two situations the LTRs cannot serve as promoters for *c-myc* and activation of either a novel *c-myc* promoter or the normal promoter must occur, presumably under the influence of the enhancer element described in restriction fragments containing avian virus LTRs (129). The initiation sites for transcription under these circumstances have not yet been mapped.

In MLV-induced T cell lymphomas of mice and rats, *c-myc* insertion mutations that forbid the use of the MLV LTR as a promoter for *c-myc* seem to be the norm. Almost all of these insertions are located within or on the 5' side of the first *c-myc* exon and directed away from the gene (43a, 217; Y. Li, N. Hopkins, G. Selton, A. Berns, personal communication), although a few are positioned downstream of *c-myc*, in one case at least 24 kb beyond the third

exon (43a). Proviral insertions with a transcriptional orientation directed away from the activated oncogenes are also commonly observed in MLV mutations of *pim* (48a) and in MMTV mutations of *int-1* and *int-2* (see below).

**ADDITIONAL MUTATIONS IN PROVIRALLY MUTATED LOCI** In the several instances in which host-viral junctions have been examined at the nucleotide level in *c-myc* insertion mutations, it appears that the accepted rules of retroviral integration have been followed: two base pairs have been removed from the ends of LTRs during insertion, and six bp at the host insertion site have been duplicated (146, 256). However, as first recognized from restriction mapping of the proviral mutagens (71, 73, 145a, 166a, 167), deletions and possibly other secondary rearrangements have altered normal proviral structure. Most commonly, these deletions remove regions of viral (and perhaps cellular DNA) encompassing the 5' LTR in proviruses located on the 5' side of the *c-myc* coding sequence in the same transcriptional orientation. There is experimental support for the claim that one effect of such deletion mutations is more efficient transcription of *c-myc* from the 3' LTR (48). However, other sorts of deletions have also been observed: proviruses on the 5' side of *c-myc* in the same transcriptional orientation have sustained a loss of their 3' ends, so that transcription of *c-myc* proceeds from the 5' LTR through viral coding domains (162) in the manner predicted to precede transduction of proto-oncogenes (see below), and proviruses in the opposite transcriptional orientation have incurred deletions that prevent expression of viral genes, e.g. by removing viral signals required in *cis* for synthesis, processing, or translation of RNA (166a, 167).

Two proviral deletion mutants have been examined by nucleotide sequencing. One retains only a single copy of the ALV LTR that lacks two bp from both ends and is flanked by a six-base duplication of cell DNA, indicating that it arose by homologous recombination between the LTRs of an initially intact provirus (256). Since the provirus in this case was inserted in the same transcriptional orientation as *c-myc*, the residual LTR is positioned to serve as promoter for a hybrid transcript that includes the coding exons of *c-myc*. The other sequenced deletion mutation occurred in a provirus (from tumor LL3) positioned in the opposite transcriptional orientation to *c-myc* (256). Although the LTRs were spared, the approximately 1 kb deletion removed the tRNA primer binding site, the RNA packaging site, the splice donor site, and the translational initiation codon for all the viral proteins. Both deletion mutations must have occurred during or after infection, since the proviruses lack sequences required in *cis* for transmission through the virus life cycle.

Nucleotide sequence alterations in *c-myc*, as well as deletion mutations in proviruses, are present in some insertionally mutated loci. Comparison of a partial sequence of the mutant *c-myc* locus from tumor LL3 with the homologous region of normal chicken *c-myc* (252) revealed three additional G residues

in the short portion of intron between the insertion site and the second exon (D. Westaway, unpublished data) and three nucleotide substitutions in the first 200 nucleotides of the second exon (256). One of these, a silent C → A transversion, was later judged to be an inherited polymorphism because the same change was observed in two other *c-myc* alleles from the same flock (D. Westaway, C. Nottenburg, unpublished data). However, a second change that does not alter the coding sequence, a G → A transition, must represent a somatic mutation because it inactivates an *Sst*I recognition site shown to exist in both *c-myc* alleles in normal tissue from the bird carrying tumor LL3. A third nearby change, a C → A transversion that converts a proline codon to a threonine codon, appears to be a somatic mutation as well, since the change is not found in alleles from the same flock that harbor the polymorphic marker.

A further genetic alteration of *c-myc* has been observed in bursal lymphomas induced by CSV (151a). Some insertionally mutated alleles are five- to ten-fold more abundant than single copy genes and the unaffected alleles are not detected. Apparent loss of normal *c-myc* alleles, presumably a result of loss of one member of a chromosomal pair, also seems to occur in some ALV-induced bursal lymphomas, perhaps accompanied by low level amplification of mutant *c-myc*, since only the rearranged allele can be identified by the Southern transfer procedure (71, 73, 94, 167).

The discovery of additional mutations—deletions, base substitutions, and amplification—at the site of proviral insertion mutations raises further questions. When do the additional mutations occur? Do they contribute to the cellular phenotype, providing a selective advantage? Since cells bearing multiple mutations within *c-myc* dominate the tumor mass, either the additional mutations must be generated at the time of (or before) the proviral insertions or they must produce a selectable change. Proviruses with deletions of the type encountered in *c-myc* loci have not been observed among the many chosen randomly for structural scrutiny (244a); however, under selective conditions (see below), various deletion mutants, including those resulting from homologous recombination between LTRs, can be recovered at low frequency (245). Thus, barring some marked stimulus to deletion formation in lymphoma cells, a selective mechanism is likely to operate. The timing of nucleotide changes in *c-myc* seems less certain, since there is insufficient information about the effect of proviral integration upon host sequences surrounding those proviruses chosen randomly for study. There appears to be concerted mutation, however, since some of the changes do not affect the coding potential of *c-myc* and seem unlikely to affect the efficiency of expression.

The selectable properties that might be conferred upon bursal cells by the additional mutations have not yet been rigorously defined. Deletion mutations within proviral DNA could mitigate the host's immune response against tumor cells by preventing synthesis of virus-specific proteins that serve as antigens on

the cell surface. Proviral deletions might also have the more intriguing effect of augmenting expression of *c-myc* (48). Amplification of insertionally mutated alleles could provide selective advantage on quantitative grounds. Lastly, nucleotide changes that alter the amino acid sequence of *c-myc* protein could render the protein more effective as an oncogenic factor.

What can be done to discern the phenotypic effects (if any) of these multiple mutations within *c-myc* alleles? Removal of the mutant alleles from the complex environment of the tumor cells to a new context would seem to be necessary to measure their effects, but to date the only convenient, direct assay for activated *myc* alleles is the co-transformation assay in rat embryo cells (114). In this assay, the crucial determinant of activity appears to be an efficient transcriptional promoter upstream from *c-myc* coding sequence in the correct orientation. Thus, normal *c-myc* exons transcribed from LTRs linked to *c-myc* in vitro are competent in the co-transformation assay, whereas *c-myc* alleles from tumors with proviral insertions in atypical arrangements are not, even when the *c-myc* gene has undergone nucleotide substitutions (W. Lee et al, unpublished data). More quantitative means to assess transformation efficiencies or the use of more appropriate cell types (e.g. B lymphocytes) may be required to elicit the consequences of the mutational events recorded in the *c-myc* alleles described above.

#### *Characteristics of Other Proviral Insertion Mutations in Proto-Oncogenes*

The several other cellular loci found afflicted by insertion mutations (Table 4) have been subjected to less scrutiny thus far than the *c-myc* alleles from bursal lymphomas. Nevertheless, a number of interesting contrasts with the activated *c-myc* alleles have come to light.

1. The insertion mutations in *c-erbB* described in ALV-induced erythroblastosis (74) seem likely to result from proviruses that are flanked on both sides by parts of *c-erbB*. This inference is based on the idea that *c-erbB* encodes the EGF receptor, a protein of approximately 138–175,000 Mr, whereas the abundant *c-erbB* protein in erythroleukemia cells is similar in size (approximately 62–75,000 Mr) and peptide composition to the product of *v-erbB* (H. Beug, H. J. Kung, personal communication), apparently representing the carboxy terminal half of the EGF receptor [(60) and references therein]. This finding suggests that the oncogenic activation of the *c-erbB* gene may depend on truncation of the protein product in both viral transductants and insertionally mutated loci. A limited analysis of an LTR-*c-erbB* junction from one erythroblastic tumor shows that the LTR is in the same transcriptional orientation as *c-erbB*, just upstream from an exon homologous to *v-erbB*, and that two bp are missing from the LTR as anticipated in a normally integrated provirus (74).

2. MMTV proviruses are about equally distributed on the 3' and 5' sides of the *int-1* locus, but all save two are directed transcriptionally away from the gene (155; R. Nusse, personal communication). A similar situation obtains in MMTV-activated *int-2* loci (56a, 170). Hence, the observed transcriptional activation of these genes normally depends on the effect of a viral enhancer rather than of a viral promoter. Two other features of *int-1* and *int-2* distinguish them from some of the other targets of oncogenic insertion mutations: first, the genes are not expressed at detectable levels in any of the normal tissues examined to date, including mammary glands from pregnant and lactating females (56a, 156; Y. K. T. Fung, unpublished data), implying that the genes may be converted from transcriptionally inactive to active genes rather than merely stimulated to higher levels of expression; second, these two genes have not been encountered among retroviral oncogenes (i.e. they are not *c-onc*'s).

The *int-1* gene is composed of four exons that contain the entire coding domain, with a relatively long (approximately 800 bp) non-coding region at the 3' end of the final exon (A. van Ooyen, R. Nusse, personal communication; G. Shackelford, T. K. T. Fung, unpublished data). Most of the MMTV insertions on the 5' side of *int-1* appear to reside upstream from the transcriptional initiation site, with the exception of two insertions in the same transcriptional orientation as *int-1* (R. Nusse, personal communication). MMTV insertions on the 3' side of *int-1* are sometimes positioned within the final exon, between the end of the coding sequence and the polyadenylation site (155); as a result, *int-1* transcripts conclude with the U3 sequence copied from the MMTV LTR. Since the U3 region is approximately 1200 bp in length (57), insertions of this type produce a transcript longer than the usual 2.6 kb mRNA. The absence of the 2.6 kb *int-1* RNA species in tumors containing the longer, allele-specific RNA confirms that activation of *int-1* functions in *cis* but not in *trans*.

Few lesions other than the MMTV insertions in mutant *int-1* and *int-2* alleles have been implicated in the activation process. Restriction mapping has not revealed overtly mutated proviruses in most cases, although a mutant *int-2* allele has been found to contain a solitary LTR, the presumptive product of homologous recombination between MMTV LTRs (G. Peters, C. Dickson, personal communication). The few host-viral junctions that have been sequenced show only the expected absence of two bp from the end of the LTR (R. Nusse, personal communication). The mutant alleles do not appear to be amplified in number, but structural or functional tests for additional mutations within the *int-1* locus have not been performed.

3. Insertions of intracisternal A particle (IAP) proviruses into the mouse *c-mos* gene have been described in cell lines derived from two BALB/c plasmacytomas, one tumor induced with mineral oil (MOPC 21) and one induced with pristane (XRPL24). In the latter case, the 4.7 kb IAP insert is

positioned within the coding sequence of the only known exon of *c-mos*, 66 codons downstream from the start of homology with one of the *v-mos* alleles in the transcriptional orientation opposite to that of *c-mos* (27, 184). The consequences of the insertion include the transcriptional activation of an oncogenic sequence that is normally silent (139), producing a 1.2 kb RNA species presumably initiated from an occult promoter and generating a transforming gene competent to cause neoplastic change in NIH/3T3 fibroblasts (184). In cell lines derived from MOPC 21 (including a popular partner in the formation of hybridomas, P3 X63 Ag8), the IAP DNA is located 58 codons closer to the 5' end of *c-mos* in the same transcriptional direction (36). In this arrangement, *c-mos* RNA is at low or undetectable levels despite the presence of a 3' LTR positioned to serve as a promoter for downstream transcription (36, 78); it is not known whether the relatively poor expression of *c-mos* in derivatives of MOPC 21 is still adequate to affect the phenotype of the plasmacytoma cell.

A striking peculiarity of the IAP insertion in XRPC24 cells warrants further comment. It is generally held that IAP proviruses are defective for production of infectious extracellular virus particles, but the intracisternal particles are thought to synthesize new IAP DNA molecules from IAP RNA and to direct its integration by retroviral mechanisms. Analysis of IAP-host junctions supports this view: two bp from the ends of LTRs appear to be missing at the junctions and six bp of host sequence are duplicated to flank the IAP DNA (27, 113). However, in the XRPC24 insertion mutation, the two IAP LTRs exhibit considerable sequence divergence, a finding inconsistent with the generation of the LTRs from sequences present only once in the RNA template for reverse transcription (244a). The marked differences between the LTRs could be explained by a high mutation rate following the insertion, by synthesis of the insertional mutagen from an IAP with heterodimeric RNA, by recombination between heterologous IAP DNAs prior to integration, or, most provocatively, by transposition of IAP DNA without passage through an RNA intermediate. More examples of newly integrated IAP DNAs or recovery of the progenitor of the XRPC24 IAP mutagen will need examining to judge among these possibilities.

4. The *c-myb* rearrangements in several plasmacytoid lymphosarcoma lines derived from Ab-MLV-infected animals occur in the absence of Ab-MLV proviruses and were originally proposed to result from insertion and deletion of proviral DNA (144). However, cloning of the affected loci has revealed the presence of helper MLV proviruses, with small internal deletion mutations, in an intron upstream from known *c-myb* exons and in the same transcriptional orientation as *c-myb* (G. Shen-Ong, D. Mushinski, M. Potter, personal communication). It seems likely that these MLV insertional mutagens, rather than Ab-MLV, were the primary determinants of tumorigenesis.

*Structural and Functional Properties of Translocation Breakpoints near Cellular Oncogenes*

GENERAL FEATURES OF *c-myc*; IMMUNOGLOBULIN GENE TRANSLOCATIONS Efforts in several laboratories to characterize the translocations in murine plasmacytoma (PC) cell lines and human Burkitt lymphoma (BL) lines that join immunoglobulin (Ig) genes to *c-myc* have yielded a rich but complex harvest [see reviews in (107, 119, 169, 191)]. Although each translocation event appears to have its interesting peculiarities best viewed by descriptions of individual cases (see below), common features have emerged from a census of the translocations examined to date.

1. Whenever t(8;14) is observed cytogenetically in a BL line, *c-myc* with distal portions of chromosome 8 is transferred next to the Ig heavy chain constant region locus on 14q<sup>+</sup>, and when t(12;15) is observed in a PC line, *c-myc* from chromosome 15 is joined to the heavy chain locus on chromosome 12. In both species, the heavy chain and *c-myc* genes are joined head-to-head in divergent transcriptional orientations. The less frequent unions of human chromosome 8 with a chromosome carrying a light chain gene [t(8;22) and t(2;8)] have only been examined in a few BL lines, but again the patterns seem constant; with t(2;8) or with t(8;22), the light chain constant regions, sometimes preceded by at least part of variable regions, appear on 8q<sup>+</sup> on the 3' side of *c-myc*, with the joined loci in the same transcriptional orientation (47, 52, 62a, 63, 230b). The role of *c-myc* in the relatively rare t(6;15) in plasmacytoma lines (96) has yet to be established at the molecular level (J. Adams, personal communication).

2. In BL lines with the less common translocations, there is concordance between the type of light chain encoded at the site to which *c-myc* is translocated and the type of light chain produced, even though the translocation chromosome contains a non-functional (allelically excluded) locus (121). There is, however, at least one exception to this rule, a BL line in which t(8;22) is accompanied by production of kappa light chains (129a).

3. In the PC lines, most breakpoints within *c-myc* map to a region of 1–2 kb that includes the first (non-coding) exon and part of the first intron. Breakpoints within the heavy chain locus have generally fallen in a switch (S) region. Most commonly, the latter sites are within a domain containing an S<sub>α</sub> region, usually S<sub>α</sub> recombined with S<sub>μ</sub> (implying that heavy class switching might have occurred prior to the translocation) or within an S<sub>α</sub> region not directly joined to S<sub>μ</sub> (44).

4. In the BL lines with t(8;14), the *c-myc* breakpoints appear to be scattered on either side of the first (non-coding) exon, far enough from *c-myc* in several instances to fail to produce altered *c-myc* restriction fragments. Most of the heavy chain gene breakpoints in BL lines are within the S<sub>μ</sub> region, although significant exceptions exist [e.g. the breakpoint in the Daudi line appears

to be within the  $V_{H3}$  region (64), and in the Raji line it is within an  $S_{\mu}$  region joined to  $S_{\gamma}$  (176)].

5. Although breakpoints within the Ig and *c-myc* loci are sometimes clustered within a few kbp, there is no evidence for homology between recombined sequences or for strict sequence specificity. The Ig breakpoints within S regions generally lie near consensus S sequences, implying that the enzymes involved in heavy chain class switches participate in translocations. Fortuitous S-like sequences may also influence the sites of recombination within *c-myc* in some cases (80b), and the sequence GAGG has been noted to lie 10–20 bp from the breakpoint in *c-myc* in five PC lines (170c).

6. The reciprocal products of translocation events can be found by molecular (if not by karyological) techniques, implying that reciprocal exchange is the norm.

7. Reciprocal translocations are imprecise, with loss of a few to a few hundred nucleotide pairs from one or both chromosomal partners. Unexpected nucleotide pairs (1–5bp) are sometimes found at junctions in the recombinational products, and in one case thus far a duplication was generated at a breakpoint. These findings suggest a general model for translocation involving staggered single-stranded cuts at the breakpoints, with polymerization or nucleolytic activity prior to ligation (80b).

8. Alterations in the nucleotide sequence of *c-myc* are common accompaniments of translocations in BL lines. Generally these are confined to the first exon and hence do not affect the amino acid sequence of the *c-myc* protein (175a, 230b, 232), but in some cases changes are also found in the second exon (176). Thus far, a limited search has not revealed significant nucleotide sequence changes in translocated *c-myc* genes in PC lines (L. Stanton, C. Croce, M. Cole, personal communication).

9. Some features of the rearranged chromosome carrying the coding region of *c-myc* suggest that *c-myc* should not be expressed: recombination inevitably occurs with the allelically excluded Ig locus, the *c-myc* gene is often deprived of its normal promoter, and the Ig components are not suitably positioned to provide a promoter for *c-myc*. Nevertheless, production of *c-myc* RNA occurs from the translocation chromosome, with little or no expression of the *c-myc* allele that remains on the normal chromosome, sometimes producing what appear to be overtly high levels of *c-myc* RNA (7, 63, 131, 150) and at other times more subtle differences [e.g. changes in differential usage of the two transcriptional initiators in the first exon (119, 232).] In a few instances (e.g. 93) such effects may be mediated by cell-specific enhancer elements identified within immunoglobulin loci (10, 81), but normally the known enhancers are relegated to the translocation product lacking *c-myc* coding exons (e.g. 176). Even in the absence of known enhancers, the expression of translocated *c-myc* genes seems dependent upon the cellular environment, since it is repressed in

hybrids between BL cells and mouse fibroblasts (149, 150). Effects of translocation upon the translation efficiency of *c-myc* mRNA have been proposed to result from deletions or base changes affecting the first exon (175a, 232), particularly since a portion of the first exon appears to be inversely repeated in the second exon (194); formation of a stable duplex might normally impede translation of *c-myc* mRNA, and an inability to form it could augment the yield of *c-myc* protein. However, direct comparisons of amounts of *c-myc* protein in BL or PC lines have yet to be published.

Many of the features of these translocations may reflect the specialized properties of genetic partners that normally undergo programmed rearrangements (the Ig genes) and of the type of cell (early or late B cells) in which the events occur. The location of chromosomal breakpoints within S regions, the loss of nucleotides at recombination sites, and the presence of somatic mutations in *c-myc* (moved to the position normally assumed by the hypermutable  $V_H$  genes) are characteristics that mirror events in the ontogeny of Ig genes (234). It remains to be determined whether translocations involving many sites occur during the development of such tumors, with selection for those that activate *c-myc*, or whether there is a strong predisposition to the kinds of translocations observed. There is little reason to suppose that external initiating factors in tumorigenesis affect the translocation process: there is no evident correlation with the agents used to induce PC tumors in mice (pristane or mineral oil) or with the presence or absence of Epstein-Barr virus in BL lines. Regrettably, translocations have yet to be described in PC or BL removed directly from the hosts.

**SPECIFIC EXAMPLES OF *c-myc*; IG TRANSLOCATIONS IN PC AND BL LINES** Properties of the best studied translocation products illustrate some of the general features of *c-myc*-Ig translocations.

1. The PC cell line X63 Ag8 (sometimes known as P3 and commonly used to generate mouse hybridomas) was derived from the tumor MOPC 21 induced in a BALB/c mouse by peritoneal injections of mineral oil. *c-myc* is joined to the heavy chain locus at sites within the first exon of *c-myc* and within an  $S_\mu$  region directly joined to  $S_{\gamma 2b}$  in a manner that forbids distinction between a translocation that preceded and one that followed heavy chain switching. Comparison of the sequences of both recombinational products with the sequences of germ line *c-myc* and IgH genes reveals that seven bp have been lost from chromosome 15 during the reciprocal translocation events; a single A:T nucleotide pair of unknown origin is present at one junction point (61, 80b, 148). In addition, 491 bp have been deleted by homologous recombination between short direct repeats on the 3' side of the switch region, although it has not been unequivocally demonstrated that this deletion occurred in the tumor cell rather than during molecular cloning. Notably, X63 Ag8 and sibling lines contain three

other candidate oncogenes described elsewhere in this essay: a *c-mos* gene activated by an IAP insertion mutation (36, 78), a transcribed gene on chromosome 10 joined to a kappa light chain locus by translocation (168), and a transforming gene unrelated to *ras* genes (117).

2. A second BALB/c-derived PC line, MPC 11, has undergone a translocation involving the first exon of *c-myc* and the  $S_{\gamma 2b}$  region; in this case, nucleotides have been lost from both chromosomes: 11 bp from chromosome 15 and approximately 300 bp from chromosome 12 (216). Short inverted repeats are present near the breakpoints in *c-myc*, but these have not been directly implicated in the translocation mechanism.

3. The PC line J558 has lost about 1 kb of *c-myc* on the 5' side of the site in the first exon that has been joined to an  $S_{\alpha}$  sequence in a  $S_{\mu} S_{\alpha}$  domain (44), and the same or a second deletion has removed at least part of  $S_{\alpha}$  (80b). The chromosome containing the *c-myc* coding region from the 3' side of the breakpoint has an extra A:T base pair at the junction, as in X63 Ag8, whereas the reciprocal product contains an extraneous four bp sequence (AACC) at the junction (80b). Sequencing of *c-myc* exons on this chromosome reveals only a single non-coding change from the germ line *c-myc* sequence (L. Stanton, personal communication).

4. The situation in the PC line W267 is similar to that in J558. Twelve bp are missing from the translocation breakpoint in the first intron of *c-myc* and about 400 bp from the breakpoint in  $S_{\alpha}$ . In addition, three extraneous bp (CTT) are present at the fusion site in the reciprocal translocation product (80b).

5. In the PC line HOPC 11, 106 bp from the breakpoint region in the first intron of *c-myc* are duplicated and present in both products of the translocation event (80b). This finding provides the best support for the claim that the translocation mechanism involves staggered cutting at the breakpoints. In this cell line, 1.6 kb have been lost from the partner site in the  $S_{\alpha}$  region of chromosome 12, and an extraneous 5 bp sequence (CCTAT) is present at the joint on the reciprocal product of translocation (80b).

6. In the cell line BL22, a site about 1 kb on the 5' side of the first *c-myc* exon is joined to  $S_{\mu}$  (12). Although the second and third *c-myc* exons do not deviate from the germ line sequence (12), the first exon displays small deletions and several nucleotide substitutions (232). Similar changes confined to the first exon—7% base changes and a 35 bp duplication—have been encountered in the translocated *c-myc* allele from the Daudi BL line, t(8;14), with a breakpoint at least 12 kb 5' of *c-myc* (175a). The amount of *c-myc* RNA is not appreciably greater in BL22 than in lymphoblastoid cell lines, but the first of the two transcriptional initiation sites is used 4–5 times as frequently as in lymphoblastoid cell lines (232).

7. The BL line, Raji, resembles BL22 in that a site about 2 kb on the 5' side of *c-myc* is joined to  $S_{\mu}$ , but the  $S_{\mu}$  region is fused with  $S_{\gamma}$  and changes in the

*c-myc* sequence are not confined to the first exon (176). In addition to several substitutions and a 10 bp deletion in the first exon, there are three short deletions in the first intron (including one that removes the splice donor site), 25 nucleotide substitutions dictating 16 amino acid changes and a three bp insertion in the second exon, and 9 nucleotide substitutions in the second intron. The third exon does not differ from published germ line sequence. In the Raji line, unlike most BL lines, the normal (untranslocated) *c-myc* locus is also transcribed (175a), prompting the suggestion that the product of the translocated locus is defective in a *trans*-active repressor function (175a, 119). It is possible that the striking changes in Raji *c-myc* are consequent to concerted mutagenic activity during heavy gene class switching or during translocation. Although normal tissue is not available from the patient in which the tumor arose, it is unlikely that these many differences are inherited polymorphisms.

8. In the BL line JBL2 with t(2;8), alterations in the structure and expression of *c-myc* are associated with translocation breakpoints at least 20 kb downstream from *c-myc* and upstream of the kappa chain constant gene. A 2.5 kb region that includes the first *c-myc* exon has been duplicated on the recombined chromosome, and the duplicated exon contains several nucleotide substitutions and a small internal duplication. The concentration of *c-myc* RNA is elevated three–five-fold over levels in lymphoblastoid lines, with one RNA species retaining sequences from the first intron and another appearing to be spliced normally (230b).

9. Nucleotide substitutions in *c-myc* have also been observed in BL lines, LY 67 and MAKU, with t(8;22) (175a). The changes are most numerous in first exon and intron, but cDNA cloned from the LY 67 line reveals a T→C transition in the second exon that dictates a change of ser-62 to pro-62 in *c-myc* protein (175a).

10. An 8;22 translocation in line BL37 has joined a site 5 kb upstream of C<sub>λ</sub> to a site 4 kb downstream of *c-myc* (98). During recombination, 21 bp were lost from chromosome 22. In this variant translocation, the joining sites bear no relationship to S regions of heavy chain loci. Despite the distance between the *c-myc* promoter and the Ig locus (> 10 kb), a marked effect on expression of *c-myc* was observed; *c-myc* RNA is fifteen-fold more abundant than in lymphoblastoid lines and the first of the two initiation sites is preferred over the normally favored second site.

11. The BL line, Manca, differs from most BL lines with t(8;14) in two respects: the breakpoint in *c-myc* is within the first intron and, more exceptional, the breakpoint in the Ig heavy chain locus is on the 5' side of S<sub>μ</sub>, placing a known enhancer element next to the coding exons of *c-myc* (93). Thus, transcription of *c-myc* may be affected by an enhancer element and translation may be augmented by the absence of sequences from the first exon in *c-myc* mRNA (194).

12. In another BL line with t(8;14), ST486, the Ig heavy chain enhancer is joined to the first exon of *c-myc* on the reciprocal translocation product, 8q<sup>-</sup> (46a, 79); short transcripts containing sequences from the first exon of *c-myc* are abundant, whereas the *c-myc* coding exons are expressed at near normal (79) or augmented (46a) levels, presumably from 14q<sup>+</sup>. Differences in regulatory mechanisms are implied by findings that expression of the first exon on the reciprocal product is suppressed in cell hybrids formed with human lymphoblastoid cells, whereas expression of the coding *c-myc* exons is suppressed in hybrids formed with mouse PC cells (46a). Both translocation products have been isolated from this line: although a simple, non-homologous joining to S<sub>μ</sub> was found 280 bp downstream from the first *c-myc* exon, restriction mapping of the reciprocal product suggested that additional undefined rearrangements had occurred (79).

**THE PH<sup>1</sup> CHROMOSOME** The piece of chromosome 9 transferred to chromosome 22 during the reciprocal translocation that generates the Ph<sup>1</sup> chromosome, a common marker in chronic myelogenous leukemia (CML) in man, is karyologically invisible and hence estimated to be less than 5000 kb in size. Detection of DNA from chromosome 9 on Ph<sup>1</sup> was initially accomplished by using a probe for the *c-abl* gene (54), and chromosomal walking later identified the breakpoint on chromosome 9 in one CML cell line to lie within 14 kb upstream of a region homologous to *v-abl* (95). The DNA specific for chromosome 22 from the 9-22 chimeric clone was subsequently used to identify a region of about 5 kb, called *bcr*, that encompasses all of the chromosome 22 breakpoints in leukemic cells obtained from 17 of 19 CML patients (87). (The two negative cases did not have karyological evidence of the 9;22 translocation.) The chromosome 22 breakpoints appear to be scattered throughout the *bcr* region, though nucleotide sequencing of the junctions has not been reported. The sites of recombination on chromosome 9 seem to be less restricted and are usually at least 40 kb upstream from known exons of *c-abl*. These findings focus attention on the possibility that the *bcr* region may have some role in leukemogenesis; although *bcr* is transcribed in at least some CML lines (J. Groffen, personal communication), there is no information about its coding potential. Similarly, the effect of the translocation upon the behavior of *c-abl* has not been fully defined. *c-abl* mRNA of atypically large size and increased abundance (27a, 38a) and a larger than normal form of the *c-abl* protein with an active tyrosine kinase (O. Witte, personal communication) have been recently observed in the CML line K562. Moreover, in the same line, the Ph<sup>1</sup> chromosome is accompanied by a modest amplification and an incompletely elucidated rearrangement of *c-abl*, with co-amplification of a resident marker for chromosome 22, the λ Ig gene (38, 95). Other Ph<sup>1</sup>-positive CML lines that do not display amplification or local rearrangement of *c-abl* also contain augmented levels of an enlarged *c-abl* RNA (38a, 27a). Neither the λ Ig gene [which is at

least 45 kb distant from *bcr* and retained on Ph<sup>1</sup> (175)] nor *c-sis* [which is far from *bcr* on chromosome 22 and transferred to 9q<sup>+</sup> during formation of Ph<sup>1</sup> (11)] is considered likely to play a direct role in CML.

**OTHER TRANSLOCATIONS AND REARRANGEMENTS IN B CELL TUMOR LINES** Perlmutter et al (168) have described a 6;10 translocation in the NS-1 line, a derivative of MOPC21, that joins sequences about 1 kb on the 5' side of the Ig kappa constant locus to an unidentified region of chromosome 10, called T<sub>K</sub>NS-1. Although there is no sequence homology at the breakpoints, there is an intriguing similarity between the involved region of the Igκ locus and a nearby region of the Igκ locus that is joined to the S<sub>μ</sub> region from chromosome 12 in a bizarre t(6;12;15) rearrangement in the PC line 7183 (242). In the latter line, the other end of the S<sub>μ</sub> domain is joined to an unidentified region of chromosome 15 (i.e. not *c-myc*). There are now some circumstantial reasons to believe that the T<sub>K</sub>NS-1 region may be functionally significant: it is rearranged in another mouse tumor cell line, the Abelson MLV-induced B cell line, 18-81; modestly amplified in X63 Ag8, another line derived from MOPC 21; expressed in the NS1 cells but not in several normal organs; and evolutionarily conserved (R. Perlmutter, personal communication).

It is possible that the effects of translocations upon gene expression can be mimicked by deletions or inversions. Weiner et al have described interstitial deletions of band D of mouse chromosome 15 that appear to augment the expression of *c-myc* (assigned to 15 D2/3), with or without rearrangements detectable by restriction mapping of *c-myc* (255). These deletions are found in Ab-MLV producing PC lines, but it is not known whether the rearrangements are directly related to virus infection.

### *Structural Features of Amplified Oncogenes*

The current picture of the molecular events that amplify proto-oncogenes is at least as fuzzy as our view of amplifications affecting other genes. Because a phenotype as simple and selectable as drug resistance cannot as yet be ascribed to oncogenic amplifications, it has not been possible to define experimental conditions to identify factors, such as inhibitors of DNA synthesis (23), that might influence the frequency with which such amplifications occur. Nor has a full structural analysis of any amplified units bearing oncogenes been attempted.

Nevertheless, a few instructive features of amplified oncogenes have been described.

1. Amplified oncogenes may exist as part of either DM chromosomes or HSRs, even within sibling lines derived from a single tumor (3, 199).
2. The crudely approximated sizes of some amplified domains bearing oncogenes—100 to 1000 kb or more—are similar to the sizes of amplified

domains bearing other recognizable genes (197a). Accordingly, additional presumably adjacent genes may be co-amplified with the implicated oncogenes, at least one other in the mouse adrenocortical tumor with amplified *c-Ki-ras* (80a) and several others in the IMR 32 line with amplified *N-myc* (111).

3. Oncogene-containing HSRs may be located in chromosomal contexts other than those in which the oncogene is normally situated. For instance, *N-myc*, a normal resident of chromosome 2p23–24, is amplified within HSRs found on a different chromosome in each of several neuroblastoma cell lines (111, 201). In a subline of the COLO320 tumor line, amplified *c-myc* (derived from 8q24) is present in an HSR situated on both sides of the centromere in a marker chromosome that also contains part of the X chromosome (K. Alitalo, C. C. Lin, unpublished data). It is possible that the identified sites are predisposed to amplify transposed DNA (e.g. after integration of one or more DM chromosomes) in a sense analogous to that observed for metabolically-selectable genes inserted at different sites in cells subjected to DNA transformation (247).
4. Amplified oncogenes may exhibit other kinds of potentially oncogenic mutations that presumably preceded an amplification step. For example, the transformation-competent, point-mutated *c-Ki-ras2* allele in the human colon carcinoma line SW480 and the translocated *c-abl* locus on a Ph<sup>1</sup> chromosome in the human CML line K562 are both modestly amplified (38, 95, 135). In a COLO320 subline bearing DM chromosomes, many of the amplified copies of *c-myc* have lost the first (non-coding) exon as a result of a rearrangement that joins a site in the first intron to DNA of unknown provenance (3; M. Schwab, unpublished data); an atypical *c-myc* RNA species presumed to represent the rearranged locus is at least as abundant as RNA from the unrearranged gene. A rearrangement of *c-erb-B* also appears to have occurred during its amplification in A431 cells (136). On the other hand, no coding changes in codons 12, 59, and 61 have been encountered in *c-Ki-ras* cDNA clones from the mouse tumor line Y1 (D. George, personal communication).
5. Although oncogene amplification is assumed to be a somatic event, direct evidence is generally lacking. In at least one patient with neuroblastoma, it has been formally proven that *N-myc* amplification is a somatic event, since a normal complement of *N-myc* genes is present in normal tissue (200).

#### FROM *c-onc* TO *v-onc*: COLLUSION OF MUTATIONAL MECHANISMS

The most flagrant examples of mutant oncogenes are to be found among the retroviral oncogenes, the transduced, multiply mutated, and highly tumorigen-

ic forms of cellular proto-oncogenes. What molecular events underwrite the transduction mechanism? What changes in the oncogenes ensue? And which changes are significant for the biological activity of the *v-onc*'s? The complete process of transduction of proto-oncogenes by retroviruses occurs too infrequently to be studied under controlled conditions. Thus, the events must be reconstructed instead from those artefacts presently available—the sequences of *v-onc*'s and *c-onc*'s—and conjectures tested against further sequence data and against attempts to recapitulate portions of the scheme under more favorable conditions than those existing naturally.

Several important differences between *v-onc*'s and *c-onc*'s have been encountered in the many comparisons now available (239).

1. *v-onc*'s appear to be truncated versions of *c-onc*'s, frequently lacking coding sequence from both ends of the transduced domain. When missing the portion encoding the amino terminus of the *c-onc* product, the transduced domain is generally expressed under viral translational signals as a fusion protein encoded in part by viral replicative genes (*gag* or *env*). Occasionally, *v-onc*'s retain the coding sequence from one or both ends of the *c-onc* progenitor (e.g. in the cases of *v-src*, *FBJ-fos*, or *v-Ha-ras*), but regulatory signals for synthesis and processing of RNA are invariably provided by retroviral sequences (including the LTRs) present on both 5' and 3' sides of oncogenic sequences.
2. All of the introns that lie between transduced exons in *c-onc*'s are absent from *v-onc*'s; but in some instances [e.g. *v-src* (226, 228), *v-myb* (108, 110) and *MC29-myc* (207, 252; C. Nottenburg, unpublished data)] there remain short regions of intron-derived sequences from the 5' side of the first transduced exon. These intronic sequences may be contiguous with the exonic sequences in the host genome and thus include an intact splice acceptor site that is used to generate both *c-onc* and *v-onc* mRNAs (as is true for *myb* and *src* genes); or the intronic sequences may be non-contiguous with exonic sequences, having been positioned upstream from a cryptic splice donor site activated during the molding of the viral oncogene (e.g. in the generation of *MC29-myc*).
3. Comparisons of the sequences of transduced genes and transducing retroviral genomes frequently display short (e.g. 4–6 bp) stretches of homology at the recombination sites on either side of the *onc* region (239).
4. Several changes in the sequence of the transduced domain are present in each *v-onc*. These commonly take the form of multiple base substitutions, including silent, conservative, and non-conservative changes; in addition, deletions of various sizes are often encountered [e.g. in the *PRCII-fps* gene (29a, 99)], and some of these shift the reading frame [e.g. in the *FBJ-fos* gene (24)].

Two central concerns arise from these comparisons: (a) what mechanisms for transduction are compatible with observed differences between *c-onc*'s and *v-onc*'s, and (b) which of the differences between *c-onc*'s and *v-onc*'s are significant factors in oncogenic potency?

### *The Favored Model for Transduction of Oncogenes*

Any transduction mechanism must recombine retroviral sequences with both 5' and 3' sites in or near the oncogene, eliminate all introns between transduced exons, and permit retention of some intronic sequences on the 5' side of the first transduced exon. Base substitutions and deletions within the *onc* domain could be explained by events that occur before, during, or after transduction and are hence less problematic.

The prevailing model for conversion of proto-oncogenes to viral transforming genes (16, 18, 226, 244, 249) recapitulates several of the mutational themes of this essay.

1. The initial event is held to be an insertion mutation. During retroviral infection, the transducing provirus is integrated on the 5' side of the *c-onc* to be captured in the same transcriptional orientation. Although direct evidence is lacking, it is attractive to presume that the insertion mutation either initiates tumorigenesis as proposed above for several retrovirus-induced tumors or at least stimulates growth; clonal expansion of the cell containing an insertion mutation appropriate to instigate transduction would markedly improve the probability that the subsequent rare events also necessary for transduction will occur.
2. The second step is proposed to be a deletion mutation that extends from a site within the provirus to a site within the oncogene. The latter site can be within either an intron or an exon, accounting for the variety of 5' end points for host-derived sequences in retroviral genomes. (Smaller deletions or none are also compatible with the model provided that the polyadenylation signal in the 3' LTR is occasionally overridden; aberrant splicing or later deletions during reverse transcription would then have to be invoked to explain the observed viral genomes.) The proposed deletion creates a hybrid transcriptional unit, one that should generate a primary transcript with sequences normally found at the 5' end of viral RNA joined to *c-onc* sequences that extend to the 3' end of the gene.
3. The hybrid RNA will be recognized as a substrate both for splicing (to remove the introns that lie between *c-onc* exons) and for packaging into virus particles.
4. To join viral sequences necessary for viral replication to the 3' side of *onc* sequences, the mechanism that facilitates high frequency recombination between retroviral genomes is invoked. Although the details of that

mechanism are still in dispute, the central events are agreed to be the formation of heterodimers during virus assembly and recombination during reverse transcription, the latter step favorably influenced by homology between the genomic subunits (33, 100, 103, 244a). Heterodimers composed of a viral-*onc* hybrid transcript and a wild-type viral RNA from another provirus in the same cell can be expected to recombine at relatively low frequency, since any homology at the 3' ends of the RNAs will be fortuitous. Occasional and irregular recombinational products have been obtained in cell culture using model substrates (82, 84), however, providing one of the few pieces of experimental support for the transduction mechanism. When partial deletion mutants of *v-*onc**-containing viruses are used to induce tumors or infect cells in cultures, the missing domain is recovered from the *c-*onc** at a modest frequency, implying that homology accelerates the process of transduction (177, 204, 249, 250). Such results are consistent with the model discussed here and with others as well. It might be possible to challenge aspects of the model more forcefully by using the few situations in which the transduction of oncogenes appears to occur in cell culture without obvious homology (178–180), by attempting to capture metabolically selectable genes, or by exploiting the recent discovery that *myc*-containing FeLV proviruses are produced in a substantial number of FeLV-associated lymphomas arising in domestic cats (124, 141, 147).

The mechanism proposed for transduction of oncogenes requires further embroidery to account for a number of retroviral genomes.

1. There are now five instances in which sequences from two apparently unlinked cellular genes have been found in single viral genomes (Table 2). This situation could arise if transduction occurred at a site of chromosomal translocation that had previously fused the two cellular domains to be transduced; alternatively, the retrovirus that arises from the capture of one host sequence might then direct transduction of a second.
2. Two peculiarities at the 3' end of *v-*src** in the genomes of RSV (123, 202, 226, 228) also require more elaborate explanation. First, the sequence that terminates the *v-*src** coding region originates outside the *c-*src** coding sequence, about 900 bp downstream from the stop codon in *c-*src**. This situation probably results from deletion formation in the host chromosome or during reverse transcription of hybrid RNA; either deletion would be facilitated by an eight bp direct repeat shown to exist at the joining sites (228). Alternatively, an aberrant splicing event could have joined the downstream sequence to the penultimate coding exon. Second, *v-*src** alleles are flanked by direct repeats of viral sequences (approximately 100 nucleotides in length). These are proposed to result from recombinational events during reverse transcription: the 3' end of the *onc* region appears to

have been joined to a viral sequence in wild-type RNA that was also present upstream of the oncogene in the viral-host hybrid RNA.

3. The several nucleotide substitutions and deletions within the transduced domains of virtually all oncogenes (18, 239) can be most simply explained as the products of the error-prone process of reverse transcription (34), with selection for any alterations that create a more effective transforming gene. However, it is conceivable that some of the observed differences in nucleotide sequence arose in the host chromosome in the early stages of transduction, augmenting the proposed neoplastic effects of the primary insertion mutations.

### *The Functional Significance of Differences between c-onc's and v-onc's*

Despite considerable experimental effort, it remains difficult to assign functional attributes to the differences that distinguish *v-onc*'s from *c-onc*'s. This is so in part because it is often difficult to control the intracellular concentration of gene products and hence to distinguish qualitative from quantitative effects, and in part because the cell types most readily employed in the laboratory are not necessarily the target cells for oncogenesis in the animal.

Several *c-onc*'s [including *c-Ha-ras1* (31), *c-mos* (20, 159), *c-fos* (137), and *c-myc* (114; W. Lee et al, unpublished data)] have oncogenic effects on cultured fibroblasts when expressed more efficiently than usual under the influence of viral promoters, even though their protein coding sequence is unaltered. Furthermore, some of these genes (and others) have been implicated in rearrangements (Tables 4–6) that appear primarily to affect gene expression, implying an oncogenic effect based on dosage. But all of the *v-onc* derivatives of these genes have multiple alteration in the coding sequence that may also be significant. Most obviously, the differences between *c-Ha-ras1* and *v-Ha-ras* include amino acid changes sufficient to render *c-Ha-ras1* transforming without the replacement of its native promoter (Table 7). Evidence for the transforming activity of normal *c-myc* under the control of an LTR has been obtained in primary rodent embryo cells in the company of a mutant *c-Ha-ras1* allele; the various *v-myc* alleles have yet to be compared to *c-myc* in a systematic fashion in the several target cells for oncogenesis by *myc* genes. There are gross differences between *v-fos* and *c-fos* alleles [the *v-fos* sequences are either fused with sequences from two other genes, *gag* and *fox*, or altered by a frameshifting deletion of 104 bp (240, 241)], and these could enhance the neoplastic potential of the genes in ways too subtle to appreciate in cultured fibroblasts. As an illustration of this possibility, the *v-abl* sequence, expressed as a *gag-abl* fusion gene in the genome of Ab-MLV, appears to require at least a portion of the

preceding *gag* domain for the transformation of lymphocytes but not for the transformation of fibroblasts (173).

The transforming activity of the *v-src* gene seems to depend primarily upon qualitative changes—either the scattered base substitutions throughout the gene or the concerted change at its 3' terminus. Expression of *c-src* at a level above that required for transformation of the same cells by *v-src* does not induce transformation (166; H. Hanafusa, personal communication), although it is possible that some high threshold for transformation by *c-src* exists. In contrast, an amount of *v-src* protein only slightly higher than that of *c-src* protein is transforming, although again the dose is important: the cellular phenotype can be radically altered with a four-fold change in *v-src* protein achieved by manipulating expression with a hormone-responsive promoter (E. Jakobovits, unpublished data).

In the aggregate, these findings suggest that full activation of proto-oncogenes through retroviral transduction may depend upon multiple changes that affect both the structure and expression of the oncogene product. Furthermore, these changes mimic many of the individual alterations described in cellular oncogenes during tumorigenesis. It is likely that retroviral oncogenes, particularly those with a long passage history, are repositories of a number of mutations that enhance their neoplastic potential and thus favor their selection under laboratory conditions. A completely satisfying analysis of the phenotypic consequences of these lesions has yet to be performed.

### INACTIVATION OF INTEGRATED *v-onc* GENES: A SAMPLER OF MUTATIONAL MECHANISMS

Most of this essay has been devoted to those mutations that activate proto-oncogenes; what can be said of mutations that cripple active oncogenes? Numerous mutations of the latter sort have been isolated during the study of viral oncogenes (125, 234a), but the vast majority of these have been generated during virus life cycles, with or without the aid of mutagens, or by manipulation of viral DNA *in vitro*. Hence, although crucial to an understanding of the functions of oncogenes, they are not directly informative about the mutations that occur in eukaryotic chromosomes and fall beyond our purview here.

In a few instances, however, mutant viral oncogenes have been isolated by seeking phenotypic revertants of virally transformed cell lines that carry only integrated viral genomes (58, 134, 219, 246). In one particularly large collection of mutants, assembled from non-transformed derivatives of a rat cell (called B31) carrying a single integrated RSV provirus, several kinds of lesions have been shown to inactivate the *v-src* gene (132, 158, 245, 246). Among

over 100 morphological revertants of B31 cells, approximately one-third have lost the entire RSV provirus, presumably by either chromosomal non-disjunction or a large deletion; about 5% have suffered deletions (1.5 kb to over 20 kb in length) that encompass the 5' LTR but spare the 3' portion of the provirus; two incurred insertion mutations following superinfection with another retrovirus lacking an oncogene (MLV); at least two mutations involved the loss or gain of a single base pair in the *v-src* coding sequence; and the rest, over half, appear to be due to nucleotide substitutions in *v-src*.

The insertion and frameshift mutations in the B31 cell line have been examined in greatest detail, in part because some of them undergo secondary mutations that restore aspects of the transformed phenotype. Both of the insertional mutagens are apparently intact MLV proviruses located between the splice donor and acceptor sites for production of *src* mRNA in the same transcriptional orientation as the RSV provirus (245). Mature transcripts from the mutant loci appear to begin in the RSV 5' LTR and end in the MLV 3' LTR; thus, *v-src* is not expressed in mRNA. One of the two insertion mutants reverts to a wild-type phenotype by homologous recombination between the MLV LTRs at a frequency of  $10^{-6}$ – $10^{-7}$  per cell per generation, leaving behind a single MLV LTR that has little apparent effect on the production of *src* mRNA. The residual MLV LTR does not appear to serve as a promoter for *v-src*; however, when retrieved from the cell and dissociated from the RSV LTRs by molecular cloning, it is competent to direct the expression of a heterologous gene lacking its native promoter (S. Ortiz, unpublished data).

Two frameshift mutations of *v-src* have been defined by nucleotide sequencing, and both conform to the dictum that loss or gain of a nucleotide pair is most likely to occur by slippage of DNA polymerase in regions containing multiple residues of a single base (221). In one case, the sequence  $GA_2G$  was converted to  $GA_3G$  (132); in the other, the sequence  $CG_5A$  was converted to  $CG_4A$  (G. Mardon, unpublished data). Both frameshift mutants revert to phenotypes resembling wild type at moderate frequency. One revertant of the +1 frameshift mutant was found to make a *v-src* protein approximately 8 kd larger than normal as a result of a spontaneous 242 bp duplication: since the duplicated region included the primary mutation and created an additional +1 shift of frame at the boundary between the two copies, three successive +1 frameshifts ultimately restored the correct reading frame (132). Two revertants of the -1 frameshift mutant result from insertion of one bp or the deletion of two bp downstream from the primary mutation, with a portion of *v-src* translated from a fortuitously open reading frame between the primary and secondary mutations (G. Mardon, unpublished data). Neither of these secondary mutations involves expansion or contraction of pre-existing multiples of a single nucleotide; in one case, the sequence  $ACTGAA$  was converted to  $ACAA$ , and in the other the sequence  $GGCTG$  was converted to  $GGCTTG$ .

## NEW DIRECTIONS TOWARD A MOLECULAR DEFINITION OF CANCER

Our main business is not to see what lies dimly at a distance, but to do what lies clearly at hand. (Carlyle)

Attending to the main business at hand, the biochemical characterization of mutations affecting cellular oncogenes in tumor cells, has provided the fuel for this essay. However, the described oncogenic mutations are no doubt limited in scope by the procedures available for discovering them; further technical and theoretical innovation may now be required to provide a satisfactory picture of oncogenesis, of the normal functions of proto-oncogenes, and of the nature of somatic mutations in vertebrate oncogenes. This penultimate section considers some prospects for the more complete view that may lie dimly at a distance.

### *Recessive Oncogenic Mutations*

All of the lesions thought to influence neoplasia and summarized to this point are presumed or documented to act in a dominant manner. However, there is abundant rationale for believing that recessive mutations contribute directly to oncogenesis. On purely speculative grounds, it is possible that cells could progress to neoplasia because they are defective in functions required for entry into an irreversible differentiation pathway or required for responsiveness to external signals that normally retard cell cycling.

Such vague possibilities take on weight in view of genetic evidence for inherited heterozygous states that strongly predispose to neoplasia in defined organs, appear to depend on a second mutation for expression (172), and have been dissociated from some of the known proto-oncogenes by linkage studies (10a). Direct support for the idea that the second mutation might involve the normal allele at the same locus on the companion chromosome has recently emerged from studies of retinoblastomas (13, 30, 142). Individuals with karyological or enzymatic signs of a small deletion mutation involving 13q14 in germinal cells develop retinoblastomas in which enzymatic assays for esterase D (a marker closely linked to 13q14) and tests for restriction site polymorphisms suggest conversion of a heterozygous to a homozygous or hemizygous state. The studies to date indicate that a number of mechanisms—including mitotic recombination, chromosomal nondisjunction with or without duplication, gene conversion, and point mutation—could account for one or another of the observed cases, although mitotic recombination seems particularly prevalent (30). These findings should stimulate efforts to isolate the posited retinoblastoma gene (Rb), to define the lesions that produce  $Rb^-/Rb^+$ ,  $Rb^-/Rb^-$ , and  $Rb^-/0$  genotypes, and to seek better definition of other loci at which recessive mutations are believed to contribute directly to cancer [e.g. the Wilms's tumor-aniridia locus on 11p13 (69)].

### *Mutations of Proto-Oncogenes in Genetically Malleable Hosts*

The only mutations of proto-oncogenes in vertebrate cells that seem presently accessible are dominant and apparently oncogenic. Such mutations seem inherently unlikely to provide much insight into the normal functions of proto-oncogenes; the phenotypic requirements may in addition impose a bias toward certain types of molecular changes (e.g. genetic rearrangements). The discovery of homologues of vertebrate proto-oncogenes, mainly *c-onc*'s, in the genomes of *Saccharomyces cerevisiae* and *Drosophila melanogaster*, traditionally favored for the induction and isolation of mutants, has enormously increased the opportunity for obtaining natural, induced, and engineered mutants in such genes.

Thus far, most of the reported studies of *c-onc* homologues in yeast and flies have been confined to structural analysis of such genes [e.g. *ras*-like genes in yeast (76, 153, 172a) and *src*- and *abl*-like genes in *Drosophila* (97, 212)], but genetic approaches are near or at fruition as well.

1. Homologous recombination during DNA transformation of yeast has been used to inactivate the two genes (*RAS1* and *RAS2*) homologous to Ha- and Ki-*ras* genes; disruptive lesions in either locus do not affect viability of haploids or diploids, but lesions in both loci prevent resumption of vegetative growth by haploid spores (104a, 230a). Similar mutation of another yeast gene less closely related to vertebrate *ras* genes (76) is also a recessive lethal (N. Segev, D. Botstein, personal communication).
2. Introduction into normal cells of a mutant yeast *ras* gene with a gly → val change in the position that corresponds to amino acid 12 in human c-Ha-*ras1* interferes with efficient sporulation (104a).
3. Existing mutants that affect progression through the yeast cell cycle seem likely in some cases to be assigned to the homologues of vertebrate proto-oncogenes. The product of the *cdc28* locus, for example, shows about 25–30% amino acid homology to several *c-onc* proteins and to cyclic AMP-dependent protein kinase (128), and the *cdc4* and *cdc36* loci show modest similarity to the host-derived sequence, *ets*, in an avian retrovirus (170b).
4. By using known deficiencies that encompass the chromosomal positions of oncogene homologues in *Drosophila*, it is possible to screen for new recessive lethal mutants likely to map within loci of interest (M. Simon, M. Hoffman, personal communication).
5. Striking variations in the mode and level of expression of the *src* and *abl* homologues occur during development of *Drosophila* (123a, M. Simon, B. Drees, personal communication), offering promise of insight into multiple effects of proto-oncogenes upon ontogeny.

### *Identifying Genes Whose Products Might Interact with Oncogene Proteins*

In other experimental systems it has proven instructive to harness genetic as well as biochemical methods to identify proteins that interact in functionally important ways (21). This is generally done by seeking extragenic suppressors of primary mutations. At least two sorts of approaches seem plausible extensions of this general strategy to oncogenes. First, viewing active oncogenes as mutant proto-oncogenes, it should be possible to isolate cells with suppressor mutations in other genes, e.g. host mutants that prevent transformation by certain retroviral oncogenes, perhaps by altering targets for critical enzymatic activities such as protein phosphorylation on tyrosine residues (40). There are accounts of mutants that might meet these criteria for papovaviral or retroviral oncogenes (e.g. 86, 101, 152, 187, 220), but the most thoroughly studied are rat lines resistant to transformation by several, but not all, retroviral oncogenes (151). Products of the restricted oncogenes—*v-Ki-ras*, *v-Ha-ras*, *v-fes*, and *v-src*—are not similar on biochemical grounds, suggesting that the mutation might affect some relatively late function in neoplastic transformation. Furthermore, analysis of somatic cell hybrids suggests, surprisingly, that resistance is dominant. These features may diminish the chances of using the mutants to identify a protein that normally comes in direct contact with a product of a restricted *v-onc* gene.

A second strategy is to isolate mutant cellular genes that suppress conditional or non-conditional mutations in active oncogenes. To date, no cellular mutants of this type have been described, although there exist mutant viral oncogenes whose behavior is host dependent. One of the mutants of the B31 *v-src* gene, for example, produces no apparent phenotypic effect in rat cells (in which the *v-src* protein displays no tyrosine kinase activity), but the same gene transforms chicken cells where its product is active as a protein kinase (158, 246). DNA transformation and molecular cloning procedures theoretically could permit the isolation of cellular genes that restore the activity of such mutant proteins.

### *Assessing Epigenetic Changes*

The findings summarized in this review further the argument that cancer has its origins in genetic rather than epigenetic change. Still, important non-mutational events seem likely to be intimately involved in oncogenic mechanisms and may be unfairly neglected (191a). Measurement of polyadenylated *c-onc* RNA is perhaps the most obvious way to begin a search for relevant epigenetic change, but even when relatively dramatic, specific, and consistent increases in *c-onc* RNA are found in the absence of overt mutations (66, 213), interpretation is uncertain. In particular, it is difficult to distinguish between effects that directly influence the oncogenic phenotype and those that simply

reflect either the transformed state or the position of the tumor cell within a cellular lineage. As experience broadens with cultured cells in many differentiated states and as the potential increases for transforming such cells with mutant oncogenes, it may be possible to distinguish between events that are critical for achieving the transformed state and those that are only consequences of it.

## A CONCLUDING PERSPECTIVE

Prior to the intensive pursuit of oncogenes, information about the structural forms of mutations in eukaryotic chromosomes was derived largely from germ line mutations of globin genes (130) and from the genetic alterations that accompany the specialized process of immunoglobulin gene development (234). Attempts to use bacterial genes introduced into cultured animal cells as easily recovered targets for somatic mutations have been compromised by the extremely high frequency of mutational events (26, 183). With molecular cloning of several cellular genes that confer metabolically selectable phenotypes in culture, somatic mutations affecting a number of genes in their normal chromosomal context have been described in molecular terms, if not usually at the level of nucleotide sequence. Among these are nucleotide substitutions and deletions affecting the *aprt* gene (211); deletions, possible translocations, amplification, and presumptive nucleotide substitutions affecting the *hprt* locus (75); and amplification and deletions affecting the *dhfr* locus (197, 238). However, the sheer number of scrutinized somatic mutations that affect proto-oncogenes cannot be rivalled in other quarters of the eukaryotic genome, and the analysis to date is certain to affect predictions about forms of mutational change throughout the genome. Although the types of mutations observed cannot be considered novel, the drama for geneticist and oncologist alike is to be found in the startling patterns: the recurrence of certain types of mutant *ras* alleles in a wide variety of tumors or the strong association of certain rearrangements with certain tumors (e.g. *N-myc* amplifications in human neuroblastomas, *int-1* or *int-2* insertion mutations in mouse mammary tumors, or *c-myc* translocations in Burkitt lymphomas). What do such observations imply about mutational mechanisms? Do some lesions occur with higher frequency than expected from random mutation? Or can all be explained by selective pressures during the growth of tumor cells?

What can be said about oncogenic mutations at present is largely descriptive; with the possible exception of proviral insertion mutations, little is known about the mechanisms by which such somatic mutations arise. It has been argued that cells en route to malignancy may be mutators and more liable to incur either nucleotide substitutions or genetic rearrangements than are normal cells or cells less advanced on a neoplastic pathway (32). Whether the observations that sustain this argument are dependent on some specialized mutational

apparatus or on more frequent action of mechanisms shared with normal cells is beyond our power to decide at present.

More importantly for an understanding of cancer, we lack clear insight into phenotypic consequences of mutations affecting proto-oncogenes. This deficiency reflects in part our imprecise definition of neoplastic change and present constraints on the ways in which we assay for it. But, as must be evident from this survey, the difficulty of assigning biological significance to mutations in tumor cells is implicit in the complexity of the neoplastic genotype, as well as the phenotype. We have seen several examples of single alleles affected by multiple mutations and of cells affected by mutations at multiple loci, although there is little reason to believe that the search for genetic change in neoplastic cells has been exhaustive. To learn which lesions initiate tumorigenic growth, which maintain it, and which lead to higher states of malignancy are tasks likely to occupy students of oncogenic mutations for many years to come.

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