

Mechanism of Carcinogenesis: The Role of Oncogenes, Transcriptional Enhancers and Growth Factors *

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«An important scientific innovation rarely makes its way by gradually winning over and converting its opponents. What does happen is that its opponents gradually die out and that the growing generation is familiarized with the idea from the beginning».

MAX PLANCK

Abstract. The oncogenes are a set of genes that have been implicated as the basis of cancer. They are the activated forms of proto-oncogenes which are part of the genetic complement of all normal cells. Activation can result from mutations in the global sense i.e. point mutations, nucleotide deletions or insertions, and chromosomal translocations. These mutations induce quantitative or qualitative changes in oncogene expression. Several human oncogenes identified in tumors and established cell lines have been cloned and studied in great detail using gene transfer techniques. Evidence has accumulated supporting the view that a single oncogene can be involved at different stages or steps in a multi-stage carcinogenesis process. Moreover, a single properly activated oncogene can trigger the whole process of malignant conversion of a normal cell. Thus both the one gene – one cancer and the multigene – one cancer hypotheses may be correct. The most frequently activated oncogenes in tumors detected by the NIH3T3 assay belong to the ras family. These ras genes code for a membrane bound protein (ras p21) which has a GTPase activity. The ras p21 encoded by the T24 activated form of the Ha-ras1 oncogene has an impaired GTPase activity. In view of the location of ras p21 and its biological effects it is proposed that the action of p21 is regulated by growth factors through their membrane receptors. Transcriptional enhancers are cis-acting positive regulatory DNA elements present in viral and cellular genomes. Their involvement in the development of certain types of cancer has been strongly suggested from studies with viruses and chromosome translocations. The *in vitro* construction of genetic hybrids linking viral transcriptional enhancers and cloned human oncogenes, and the subsequent transformation of early passage cells has been helpful in delineating stages in the malignant conversion of normal cells

and gaining insights into the mechanism of carcinogenesis. Transforming growth factors (TGFs) are low molecular weight proteins that reversibly induce anchorage independent growth of certain cells such as the NRK cells. At least two types of TGFs, α and β have been identified. Introduction and expression of cloned human Ha-ras genes in mammalian cells trigger TGF release into the medium. This can occur both in stable transformants and in cells shortly after transfection. The latter suggests that TGF release by the transfected cell is the direct result of the oncogene action rather than a consequence of a cellular change during the process of transformation. Thus the normal role of the ras gene products may be central to cell growth regulation, providing a link between the response to exogenous growth factors through their receptors and the synthesis of factors which may stimulate growth in an autocrine or exocrine fashion.

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* Dedicated to Professor Angus F. Graham on the occasion of his 69th birthday.

Key Words: Carcinogenesis, oncogenes, transcriptional enhancers, growth factors.

1. Introduction

Experiments which I first reported in 1977 at Toronto, demonstrated that the transformed phenotype of cancer cells could be transmitted from one cell to another by gene transfer. The recipient Syrian or Chinese hamster cells became anchorage independent and tumorigenic (1,2) or immortalized (3). These results demonstrated that certain transforming sequences or cancer genes act dominantly in recipient cells. Subsequently, several scientists were able to follow up these ideas and the technological approach which I first developed using the calcium phosphate technique of Graham and van der Eb (4) to clone the genes responsible (for a review, see Reference 5). These transforming DNA sequences or cancer genes were more recently named oncogenes since the first one cloned, the T24 Ha-*ras* 1 oncogene, from a human bladder carcinoma derived cell line was found to be homologous to a retrovirus Harvey murine sarcoma virus transforming oncogene (6 - 8). However, other transforming genes like the N-*ras* (9), B-*lym* (10), and *met* (11) which have been identified by transfection studies do not have counterparts in any known retroviral oncogenes. Thus, some cellular oncogenes have escaped capture by retroviruses underlining the importance of the gene transfer approach. Although some retrovirologists have placed strong emphasis on the identification of cellular oncogenes through the study of viral agents (12), it should be remembered that the definition of cellular oncogene must ultimately be based on the demonstration that the cellular counterparts themselves have transforming potential and not merely sequence homology with the viral genes.

The use of cloned oncogenes has contributed significantly to our understanding of the mechanism of carcinogenesis. Experiments from our laboratory have demonstrated that when a single properly-activated oncogene is introduced into a normal cell, it is sufficient to trigger the whole process of malignant conversion; both the quantitative and the qualitative aspects of oncogene activation are important in the transformation process (13, 14, 15). The early assumption that the Ha-*ras* or *myc* oncogenes can only be activated at a certain step or stage during this process (16,18) has been disproved by the fact that these oncogenes have now been found to act at early (14, 19, 20, 21, 22, 23, 24), intermediate (14, 25, 26, 27) or late (14, 28, 29, 30, 31, 32) stages in the pathway to cancer.

The separate areas of growth control involving oncogenes and growth factors have now been brought together by the discoveries that the platelet-derived growth factor (PDGF) is encoded by the *sis* oncogene (33, 34) and the epidermal growth factor (EGF) receptor is encoded by the *erbB* oncogene (35). The property of cancer cells to release and respond to their own growth factors (autocrine stimulation), postulated by Sporn and Todaro (36), has played an important role in linking oncogene and growth factor research. The demonstration that exogenous oncogenes can trigger transforming growth factor (TGF) release strengthens the relationship between oncogenes and growth factors in bringing about autonomous cell growth (26).

The discovery of positive (37, 38) and negative (39, 40) transcriptional regulatory elements in viral and cellular genomes coupled with the demonstration (14, 27, 41) of how (by altering the expression levels of oncogenes) they can influence the transformed phenotype of cells has added a further dimension to our views about the development of cancer.

Finally, the tissue specific expression of certain oncogenes and the ubiquitous expression of others (22, 23, 42, 43, 44, 45, 46) has advanced our understanding of the role of oncogenes in cell differentiation and the mechanism of carcinogenesis.

In this paper, I review some recent experiments bearing on the mechanisms of carcinogenesis, describe some unpublished data, and propose a model for the activation of *ras* oncogenes. (An account of my earlier work in this field can also be found in a previous publication (47).)

2. Multistage carcinogenesis

The cancer phenotype consists of several distinct characters such as growth factor or hormone independence, anchorage independence, invasiveness and metastasis (for a review, see Reference 48). Much of the evidence comes from epidemiological studies (49) and experimental chemical carcinogenesis *in vivo* (50) and *in vitro* (51). The pathway from a normal to a malignant cell is depicted in Figure 1. According to this model, the first stage, initiation, is induced by mutagens (carcinogens, radiation, viruses) and is irreversible. The second stage, promotion, can be brought about by agents such as phorbol esters. However, it should be noted that viruses can also act as promoting agents. The third stage, progression, is characterized by the occurrence of heterogeneity in the tumours. I propose that oncogenes are activated at all three stages in this pathway.

The role of individual oncogenes in multistage carcinogenesis is not completely understood and the stage-specific action of a few of them has been the subject of controversy (52, 53, 54). However, there is now strong evidence that the *ras* genes may be activated at all three recognizable stages of carcinogenesis. Thus, Ha-*ras* is activated at early stages in carcinogenesis (14, 19, 20, 22, 23, 24), intermediate (14, 26, 27) as well as late (14, 28, 29, 32). The same would seem to apply to the *myc* oncogene where its involvement in early (21) intermediate (25, 26) and late (30) stages has been observed. It has also been found that another member of the *myc* family, the N-*myc* oncogene, is amplified in metastatic neuroblastomas (31). The association of *myc* with specific chromosomal translocations in murine and human B cell tumours also argues for a role in late tumorigenesis (55). Thus oncogenes cannot be strictly placed into categories or «complementation» groups according to the step in which they might act during carcinogenesis. Another, emerging principle is the apparent lack of tissue specificity of some oncogenes, e.g. *ras* oncogenes have been found to be activated in a variety of tissues (for a review, see Reference 20).

There also exists a variety of mechanisms for the activation of cellular oncogenes. Such mechanisms include: insertional

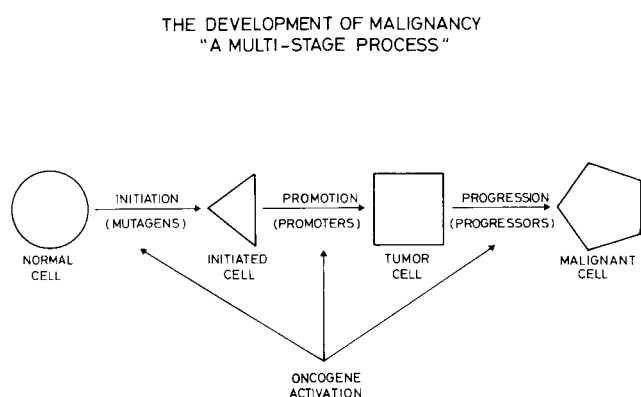


Figure 1. The multistage nature of cancer. Substances involved in the activation of oncogenes in the initiation, promotion, and progression stages are called *mutagens*, *promoters*, and *progressors*, respectively.

mutagenesis (56), point mutation (57, 58, 59), chromosomal translocation (55), gene amplification (60, 61, 62), viral transduction (63), and trans-activation (64). Most of these mechanisms are outside the scope of this article and, thus, will only receive minimal attention.

Elevated expression of proto-oncogenes induced by other unknown mechanisms might also be important in the development of cancer. Increased transcript levels in premalignant and malignant tissues have been reported by a number of investigators (22, 23, 42, 43, 44, 45, 46). The significance of these findings cannot be completely assessed at present, however, since the crucial threshold levels have not been determined. Similarly, the observation that *Ha-ras* was found to be expressed at elevated levels in regenerating liver (65) could have different implications for the mechanism of *ras* gene involvement in the process of carcinogenesis.

3. Mechanism of *ras* oncogene activation

The *ras* gene family is composed of three members designated Harvey, Kirsten-, and N-*ras* on the basis of homology with the two sarcoma virus oncogenes and an oncogene identified by transfection assays, where it was first found to be activated in some human tumor cell lines. The protein products of all three types of *ras* genes are immunologically related, have a molecular weight of approximately 21,000, and are termed p21 proteins (for a review, see Reference 5).

Comparison of the nucleotide sequence of human *ras* oncogenes compared with their normal cellular counterparts has revealed two «hot spots» for activation: codon 12, located in the first exon and codon 61, located in the second exon. A single point mutation which substitutes a different amino acid for glycine (codon 12) or for glutamine (codon 61) is responsible for the transforming properties of *ras* genes in NIH3T3 cells (for a review, see Reference 20).

Ample evidence has now been accumulated suggesting that

both qualitative (14, 57, 58, 59) and quantitative (14, 27) changes are necessary for *ras* oncogene activation. Our results suggested that at low levels of expression of the T24 *Ha-ras 1* oncogene or high levels of expression of the normal *Ha-ras 1* proto-oncogene in rodent cells, immortalization or anchorage independence and tumorigenicity could be obtained in a single-step. However, high levels of expression of the T24 oncogene could trigger complete malignant transformation of early passage rodent cells (13, 14, 15).

Strong evidence concerning the role of *ras* oncogenes in cancer is based on chemical carcinogenesis studies. Transforming *ras* oncogenes have been found in more than 70% of chemically induced tumors in different animal model systems (19, 20, 66, 67, 68). Recently, more direct evidence has been obtained that the mutational event responsible for the malignant activation of the *Ki-ras* gene in a human lung carcinoma is specifically associated with tumor development (69).

4. Possible functions of *ras* p21

A number of the oncogene encoded proteins have been studied in great detail. They are located at different sites within the cell and found to have different functions (70). For example, the gene product of the *Ha-ras 1* proto-oncogene protein p21, is localized to the inner side of the cell membrane. It binds guanine nucleotides and has a GTPase activity (71, 72). Moreover, the p21, derived from the T24 *Ha-ras 1* oncogene, which is altered in amino acid 12, has an impaired GTPase activity (71, 72). However, it has been established that other cellular proteins, designated «G proteins», have, like p21, guanine nucleotide binding capacity, GTPase activity, and function as intracellular transducers of growth regulatory signals from cell surface receptors (73). It has also been recently shown that in yeast, *ras* proteins are controlling elements of adenylate cyclase activity (74) and that mammalian and yeast *ras* genes are functionally homologous (75). From these observations, I propose the following model to describe this type of interaction (Figure 2). This model predicts that p21 mediates a signal to intracellular targets after it has received another signal from a growth factor through its receptor. A relevant model has recently been put forward by Newbold (76). It is interesting to note in this connection that EGF, which has the same receptor as a TGF, stimulates p21 to bind GTP (77). However, the precise target(s) in the cell at which p21 acts remain speculative at present although some recent experiments have suggested that the protein product of the *Ha-ras* proto-oncogene is required for initiation of the S phase of the cell cycle in NIH3T3 cells (77). Whether p21 *ras* operates through such a mechanism remains to be established.

5. Transcriptional enhancers and their role in oncogenesis

Enhancers were initially discovered in viral genomes such as of SV40 (37, 38), polyoma (79, 80), bovine papillomavirus (81, 82), retroviruses (83, 84, 85), and Herpes simplex virus (86).

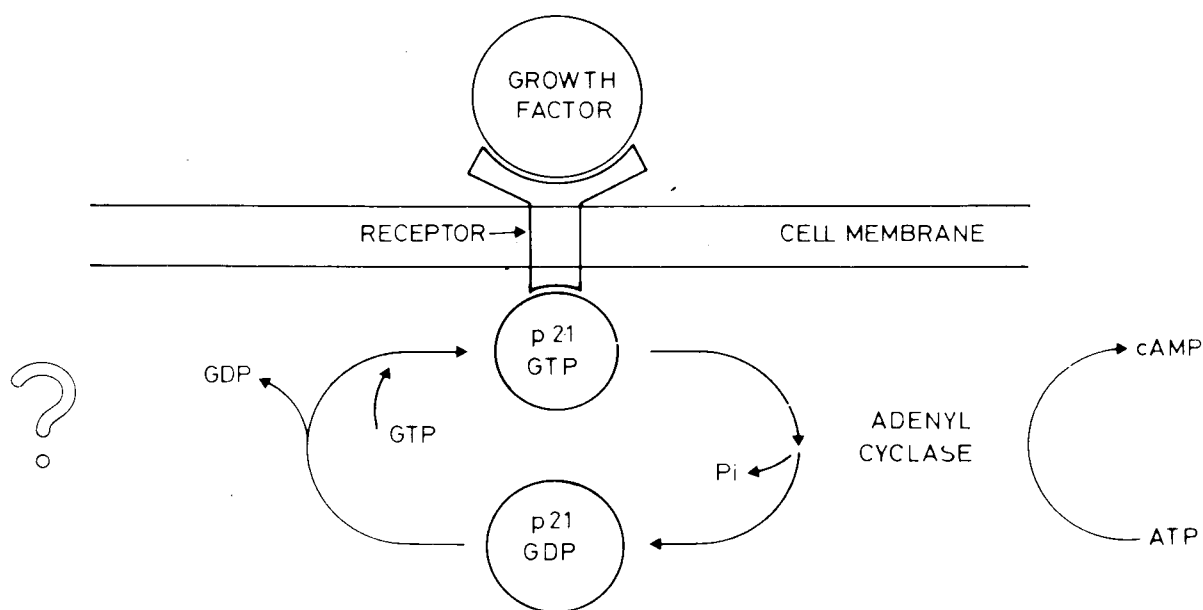


Figure 2. Hypothetical signal transmission from growth factor to adenyl cyclase through p21 and growth factor receptor.

Table I. Some properties of transcriptional enhancers.

1. Relatively short sequences
2. Cis Acting
3. Independent of orientation
4. Effective over long distances
5. Cell type and species specific
6. Consensus sequence: GXTGTGG $\begin{matrix} \text{AAA} \\ \text{TTT} \end{matrix}$

Some of their properties are summarized in Table I. Cellular enhancers have also been found in the mouse Ig heavy chain (87, 88, 89) and in the Ig K light chain (90) loci. Similarly, tissue specific enhancer elements have been described in the human Ig heavy chain (91) and Igλ light chain (92) loci. Although cellular enhancers might have a role in cell differentiation (87), they are also thought to be important in the development of cancer (14, 93, 94). In relation to this it has been shown that in certain Burkitt's lymphoma cell lines translocations of the cellular *myc* oncogene close to the heavy chain enhancer results in elevated *myc* gene expression (91). However, more direct evidence comes from experimental model systems where a retrovirus LTR enhancer is inserted next to a cellular proto-oncogene and causes elevated transcription of the gene. This mechanism of carcinogenesis is called insertional mutagenesis or enhancer insertion (initially, promoter insertion (56)).

6. Studies on the mechanism of carcinogenesis using cloned oncogenes and transcriptional enhancers

The experiments described below together with those previously published (13, 14, 15) are aimed at elucidating the mechanism of carcinogenesis using gene transfer techniques.

The introduction of defined oncogenes with or without transcriptional enhancers into cells at different stages in the pathway to cancer and the analysis of the resultant cells undoubtedly represents a powerful approach.

7. Construction of recombinant plasmids carrying cloned oncogenes

To introduce cloned oncogenes into early passage mammalian cells a dominant selectable marker, aminoglycoside phosphotransferase (*aph*), was covalently linked to the vector DNA. The *aph* confers resistance to the antibiotic geneticin (G418), an inhibitor of protein synthesis. Therefore cells which take up and express the *aph* gene survive in the geneticin containing selection medium. The construction and characterization of recombinant plasmids carrying the *aph* gene in vectors containing no transcriptional enhancer, one enhancer (from the SV40 virus), or two enhancers (from the SV40 and the MoMSV viruses), and the normal or the T24 derived Ha-*ras* 1 oncogene have been previously described (13, 14) and are depicted in Figure 3.

Recently, I have constructed recombinants carrying the human N-*ras*, derived from the HT1080 fibrosarcoma cell line (95), the normal human *c-myc* (96) and the avian retrovirus MC29, v-*myc* (97) oncogenes linked to the *aph* gene and the MoMSV enhancer. These plasmids are shown in Figure 4.

8. Introduction of recombinant DNAs into early passage mammalian cells

Using the calcium phosphate technique I have introduced these recombinants in early passage mammalian cells from

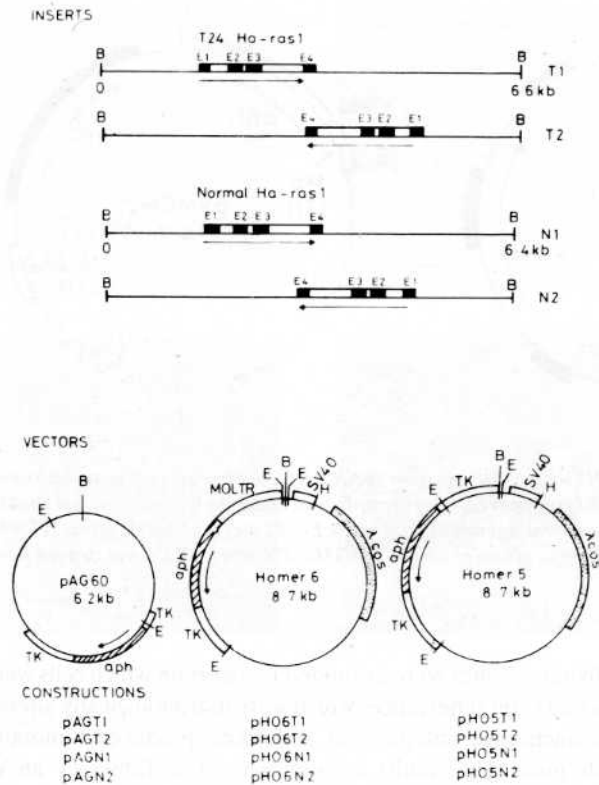


Figure 3. Recombinant plasmids carrying the T24 *Ha-ras1* oncogene (pAGT1, pAGT2, pH06T1, pH06T2, pH05T1, and pH05T2) or the normal *Ha-ras1* proto-oncogene (pAGN1, pAGN2, pH06N1, pH06N2, pH05N1, and pH05N2). The 6.6 kb BamHI fragment containing the human T24 *Ha-ras1* or the 6.4 kb BamHI fragment containing the normal human *Ha-ras1* proto-oncogene were inserted into the single BamHI sites of plasmids pAG60, Homer 6, and Homer 5 as previously described (13, 14, 15).

various species such as the Chinese and Syrian hamster, rat and human as well as in different tissues from a single species i.e. rat muscle, skin, lung, kidney, and embryo cells. I have also introduced these recombinants into already immortalized cells. Some of these results have been previously published (13, 14, 15). Here I intend to report on the transformation of Syrian hamster and human fibroblast cells. The results are summarized in Table II. Geneticin resistant colonies were obtained with both types of cells with all recombinants tested. Representative morphologies of Syrian hamster and human cells are shown in Figures 5 and 6, respectively. Table II shows the results obtained on the introduction of 1 μ g of recombinant plasmid into second passage Syrian hamster muscle (SHM) or third passage human fetal fibroblast (GM468) cells. Geneticin-resistant colonies were obtained with all recombinants in SHM, but only with recombinants carrying enhancers (Homer 6, pH06N1, and pH06T1) in human cells. However, the transformation frequencies were approximately 8 fold higher with the Syrian hamster cells (81 colonies/ μ g pH06T1 DNA/ 2×10^5 cells plated) compared to the human cells (12 colonies/ μ g

pH06T1 DNA/ 2×10^5 cells). Microscopic examination of the colonies revealed the following results: SHM cells transfected with pH06T1 (carries the mutated T24 *Ha-ras1* gene and the SV40 and MoMSV enhancers) were morphologically transformed, that is they contained highly refractile, pebble-shaped cells which grew in disoriented manner and were not contact inhibited (Figure 5). These properties were similar to those found for Chinese hamster and Wistar rat cells transformed with the pH06T1 recombinant as previously reported (14). Moreover, as presented in Table II these cells grew in methocel containing medium and therefore were anchorage independent. However, SHM cells transfected with all other recombinants tested had a flat morphology, exhibited contact inhibition and were anchorage dependent. These results are consistent with previous findings with Chinese hamster and Wistar rat cells (13, 14). In contrast, geneticin resistant human cells obtained with Homer 6 or pH06N1 showed no obvious morphological transformation although those obtained with pH06T1 grew to higher cell densities and exhibited slightly different morphology (Figure 6). Nevertheless all human cells were anchorage dependent and senesced after 70 - 80 cell doublings.

9. Introduction of oncogenes into immortalized rodent cell lines

Mouse NIH3T3 (97), rat 208F (98), and BHKC13 (1,99) cells were employed in these studies, the results of which are shown in Table III. Geneticin-resistant colonies were obtained with all recombinants in all 3 types of cells. Transformation frequencies were 10 - 20 fold higher with recombinants carrying enhancers. The highest transformation frequencies were obtained with NIH3T3 cells (16500 colonies/ μ g pH06T1 DNA/ 2×10^5 cells) and the lowest with 208F cells (790 colonies/ μ g pH06T1 DNA/ 2×10^5 cells). BHK C13 cells gave intermediate values for transformation frequency (5400 colonies/ μ g pH06T1 DNA/ 2×10^5 cells). It was noted that the transformation frequencies with the immortalized cell lines were approximately 2 orders of magnitude higher than with primary cells (see Tables II and III and Reference 13). Microscopic examination of the colonies reveals the following: all 3 types of cells transfected with recombinants carrying a T24 *Ha-ras1* gene (pAGT1 and pH06T1) exhibited a morphologically transformed phenotype whereas 208F and BHK C13 cells transfected with recombinants carrying the normal *Ha-ras1* proto-oncogene (pAGN1 and pH06N1) were morphologically normal. NIH3T3 cells transfected with pH06N1 but not with pAGN1 or pMCGM1 exhibited morphologically altered phenotypes. However, all types of cells transfected with pAGT1, pH06N1, pH06T1 and pMCGM1 were anchorage independent and tumorigenic (see below). The results with the pH06N1 in 208F and BHK13 cells were the most surprising and suggested that lack of morphological transformation as usually demonstrated by focus assay may not always be sufficient to determine anchorage independent and tumorigenic conversion. However, when the normal *Ha-ras* gene is under the

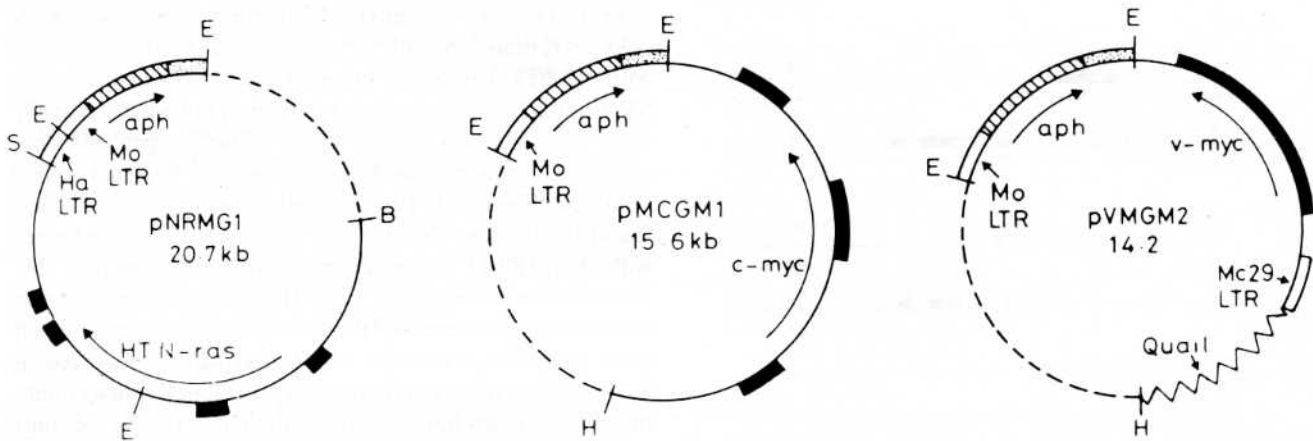


Figure 4. Schematic representation of recombinant plasmids carrying the human HT1080 N-ras oncogene (pNRMG1), the human myc proto-oncogene (pMCGM1), and the avian retrovirus myc oncogene (pVMGM2). A 2.9 kb EcoRI fragment carrying the aph gene under the 5' transcriptional control sequences of the MoMSV LTR and the 3' polyadenylation signals of the HSV-1 tk gene was inserted into the single EcoRI sites of plasmids pNras HT1080 (95) to construct plasmid pNRMG1, pMC41/C1 (96) to yield pMCGM1, and pBR326 to obtain plasmid pVMGM2. Plasmid pBR326 was derived from pBR325 (97) by deleting a 2.2 kb HindIII fragment.

influence of a strong promoter, i.e. the metallothionine promoter it can produce morphological transformation of rat 208F cells (my unpublished results).

10. Phenotypes of transformed cells

Clones of transfected Chinese and Syrian hamster, rat and human cells were propagated *in vitro*. The phenotypes of

individual clones were examined to ascertain which cells were rescued from senescence, which were morphologically altered and anchorage independent and which produced tumors in nude mice. The results are summarised in Tables IV and V. Geneticin resistant hamster and rat colonies derived after transfection of early passage cells with recombinants carrying the T24 Ha-ras 1 oncogene in the presence of one or two

Table II. Transfection of early passage Syrian hamster (SHM) and human (GM468) cells with aph recombinant plasmids^a.

Recipient Cells	Donor DNA ^b	Exogenous oncogene	Enhancers	Geneticin resistant colonies/flask or plate AV±SD	
				liquid medium	semisolid medium
SHM	pAG60	-	-	2.2± 1.4	0
	pAGN1	Ha-ras1	-	2.4± 1.3	0
	pAGT1	T24 Ha-ras1	-	2.5± 1.9	0
	Homer 6	-	SV40,MoMSV	72 ±12	0
	pH06N1	Ha-ras1	»	81 ±14	0
	pH06T1	T24 Ha-ras1	»	81 ±12	74±15
	Salmon	-	-	0	0
GM468	pAG60	-	-	0	0
	pAGN1	Ha-ras1	-	0	0
	pAGT1	T24 Ha-ras1	-	0	0
	Homer 6	-	SV40,MoMSV	8.3± 3.0	0
	pH06N1	Ha-ras1	»	10 ± 3.2	0
	pH06T1	T24Ha-ras1	»	12 ± 3.5	0
	Salmon	-	-	0	0

^aTransfection of second passage Syrian hamster muscle (SHM) or third passage human fetal fibroblast (GM468) cells was carried out using the calcium phosphate technique (4). One µg of each donor DNA mixed with 10 µg salmon DNA as carrier were added per 25 cm² flask containing 2X10⁵ cells plated the day before and geneticin resistant colonies were obtained as described (14). The results are derived from six flasks or plates per donor DNA from three experiments.

^bThe constructions of donor DNA molecules are described in Figures 3 and 4.

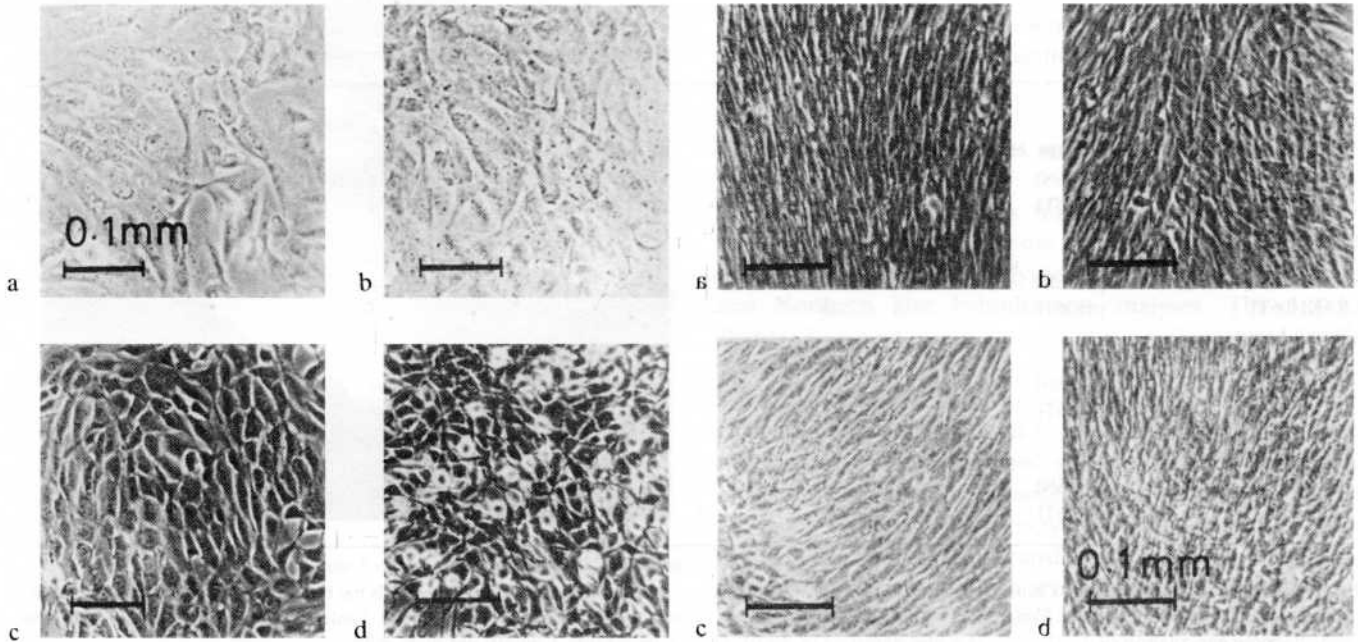


Figure 5. Normal and transformed Syrian hamster muscle cells. (a) second passage Syrian hamster cells; (b), (c), and (d) cells transfected with Homer 6, pH06N1, and pH06T1, respectively.

Figure 6. Normal and transformed human fibroblast cells. (a) third passage human fetal fibroblasts; (b), (c), and (d) cells transfected with Homer 6, pH06N1, and pH06T1, respectively.

Table III. Transfection of immortalized rodent cell lines with *aph* recombinant plasmids^a.

Recipient Cells	Donor DNA	Exogenous oncogene	Enhancers	Geneticin resistant colonies/flask or plate AV±SD	
				liquid medium	Semi-solid medium
NIH3T3	pAG60	-	-	8.5±4.1	0
»	pAGN1	Ha-ras1	-	10 ± 3.7	0
»	pAGT1	T24Ha-ras1	-	11 ± 4.5	7.0± 2.1
»	Homer 6	-	SV40,MoMSV	122 ±25	0
»	pH06N1	Ha-ras1	»	130 ±31	112 ±20
»	pH06T1	T24 Ha-ras1	»	165 ±23	121 ±15
»	pMCGM1	<i>c-myc</i>	MoMSV	133 ±18	116 ±17
208F	pAG60	-	-	4.3± 2.7	0
»	pAGN1	Ha-ras1	-	4.8± 2.2	0
»	pAGT1	T24 Ha-ras1	-	4.7± 2.3	3.1± 1.3
»	Homer 6	-	SV40,MoMSV	76 ±15	0
»	pH06N1	Ha-ras1	»	84 ±14	83 ±11
»	pH06T1	T24 Ha-ras1	»	79 ± 8.8	73 ±10
»	pMCGM1	<i>c-myc</i>	MoMSV	66 ± 9.4	52 ± 6.7
BHKC13	pAG60	-	-	2.1± 1.0	0
»	pAGN1	Ha-ras1	-	2.5± 1.2	0
»	pAGT1	T24 Ha-ras1	-	2.2± 1.2	2.0± 1.3
»	Homer 6	-	SV40,MoMSV	47 ± 7.2	0
»	pH06N1	Ha-ras1	»	43 ±8.4	42 ± 7.5
»	pH06T1	T24 Ha-ras1	»	54 ±10	36 ± 5.4
»	pMCGM1	<i>c-myc</i>	MoMSV	35 ± 6.4	50 ± 6.9

^aTransfection of mouse NIH3T3, rat 208F, and Syrian hamster BHKC13 cells was carried out as described in the legend to Table II. The amount of donor plasmid DNA was as follows: NIH3T3 cells, 10ng; 208F cells, 100 ng; and BHKC13 cells, 10ng.

Table IV. Phenotypes of transfected early passage mammalian cells.

Cells	Transfected with	Rescued From senescence	Morphologically transformed	Anchorage Independent	Tumorigenicity
Chinese hamster					
CHLF3	-	-	-	-	-
FAGT1	pAGT1	+	-	-	-
FH06N1	pH06N1	+	-	-	-
FH06T1	pH06T1	+	+	+	+
Rat					
RSK	-	-	-	-	-
RSKH06N1	pH06N1	+	-	-	-
RSKH06T1	pH06T1	+	+	+	+
Syrian hamster					
SHM	-	-	-	-	-
SHMH06N1	pH06N1	+	-	-	-
SHMH06T1	pH06T1	+	+	+	+
Human					
GM468	-	-	-	-	-
HF8H06N1	pH06N1	+	-	-	-
HF8H06T1	pH06T1	+	-	-	-

Transfection of third passage Chinese Hamster lung (CHLF3) and second passage Wistar rat skin (RSK) cells has been described in detail before (14), whereas for the transfection of Syrian hamster muscle (SHM) and human fetal fibroblasts (GM468) see Table II. Tumorigenicity was tested by subcutaneous inoculation of 1×10^6 cells (rat and hamster) or 1×10^7 cells (human) into 1 month old nude mice.

enhancers were morphologically altered, anchorage independent and produced tumors in nude mice after subcutaneous injection. The tumors grew rapidly and in some cases gave metastatic infiltrations of other organs. Injections of rat cells transformed with pH06T1 or pH06T2 into 3-week old Wistar rats also induced rapidly growing tumors (Figure 6). Cells transfected with pH06N1 were not tumorigenic.

Moreover, whereas hamster and rat cells transfected with

pH06N1 were immortalized, (they have been grown in culture for more than 200 cell doublings), human cells transfected with pH06N1 or pH06T1 senesced at 70 - 80 cell doublings and were not tumorigenic in nude mice (Table IV). Although it has recently been claimed that human bronchial epithelial cells can be transformed with a single oncogene, viz the Harvey murine sarcoma virus *ras* oncogene (101), transformation of human fibroblasts has been unsuccessful in my

Table V Phenotypes of transfected immortalized rodent cells^a.

Cells	Transfected with	Rescued From senescence	Morphologically transformed	Anchorage Independent	Tumorigenicity
Mouse					
NIH3T3	-	+	-	-	-
MN3AGNI	pAGNI	+	-	-	-
MN3AGTI	pAGTI	+	+	+	+
MN3H06N1	pH06N1	+	+	+	+
MN3H06T1	pH06T1	+	+	+	+
Rat					
208F	-	+	-	-	-
RFAGNI	pAGNI	+	-	-	-
RFAGTI	pAGTI	+	+	+	+
RFH06N1	pH06N1	+	-	+	+
RFH06T1	pH06T1	+	+	+	+
Syrian hamster					
BHKC13	-	+	-	-	-
BAGNI	pAGNI	+	-	-	-
BAGTI	pAGTI	+	+	+	+
BH06N1	pH06N1	+	-	+	+
BH06T1	pH06T1	+	+	+	+

^aSee legend to Table IV.



Figure 7. Tumor bearing Wistar rats 3 weeks after subcutaneous injections with 1×10^6 RSKH06T1 cells.

experience over the years (51). Likewise others have been unsuccessful in transforming human cells with cloned oncogenes (102).

The phenotypes of transfected immortalized cell lines (Table V) were similar for all three types of cells. As noted above the most interesting finding was that morphologically

normal cells obtained after transfection of 208F or BHK C13 cells with pH06N1 were found to be anchorage independent and tumorigenic. The implications of which were discussed above.

11. Expression of exogenous oncogenes

The Chinese hamster cells transformed with the *Ha-ras1* oncogene were studied in more detail. The level of *Ha-ras1*-specific transcripts was analyzed by RNA spot hybridization and Northern blot hybridization analyses. The 6.6 Kb BamH1 fragment of the cloned T24 *Ha-ras1* gene was used as a probe. Representative results are shown in Figure 7. The 1.2 Kb *Ha-ras1*-specific RNA was present in the transformed cells. However, much higher levels of the 1.2 Kb RNA were detected in cells transformed with pH06T1 containing the SV40 and the MoMSV enhancers than in cell lines obtained with pAGT1 which lacks a known enhancer. Moreover, when the pH06T1 and pH06T2 transformed cells were injected in nude mice the tumors which arose also contained high levels of the *Ha-ras1* transcripts. Similar results were obtained using the RNA spot hybridization assay (14) clearly demonstrating a correlation between oncogene expression and the transformed phenotype.

12. Heterogeneity of tumor cells

Cellular diversity or heterogeneity has been observed in a

Table VI TGF release by rodent cells transfected with recombinants carrying oncogenes ^a.

Cells	Transfected with	Exogenous oncogene	Enhancers	Anchorage independence	Tumorigenicity	TGF release
CHL (F3)	-	-	-	-	-	1.5 ± 1.1
FH06N1	pH06N1	<i>Ha-ras1</i>	SV40, MoMSV	-	-	14 ± 32
FH06T1	pH06T1	T24 <i>Ha-ras1</i>	»	+	+	850 ± 82
RSK	-	-	-	-	-	1.3 ± 1.2
RSKH06N1	pH06N1	<i>Ha-ras1</i>	SV40, MoMSV	-	-	17 ± 5.1
RSKH06T1	pH06T1	T24 <i>Ha-ras1</i>	»	+	+	720 ± 73
SHM	-	-	-	-	-	1.7 ± 1.4
SHMH06N1	pH06N1	<i>Ha-ras1</i>	SV40, MoMSV	-	-	12 ± 3.8
SHMH06T1	pH06T1	T24 <i>Ha-ras1</i>	»	+	+	530 ± 60
208F	-	-	-	-	-	3.2 ± 1.0
RFNRMG1-1	pNRMG1	HT1080 <i>N-ras</i>	MoMSVC, HaMSV	+	+	1540 ± 160
RFMCGM1-1	pMCGM1	<i>c-myc</i>	MoMSV	+	+	1120 ± 180
RFVCM2-1	pVMGM2	<i>v-myc</i>	MoMSV, MC29	+	+	1230 ± 150

Cells were seeded (at 2×10^6 cells/75 cm² flask) in Ham's SF12 medium containing 15% FCS. The medium was removed after 4 h and replaced with 20 ml medium containing 1% FCS. Flasks were incubated at 37°C for 3 days, the supernatants were removed and the cell count was noted. The supernatants were filtered through 0.45 μ Millipore filter and assayed for TGF activity using NRK cells. NRK cells were resuspended at 10^4 /ml in 1.2% methocel in Ham's SF12 medium containing 10% FCS. The cell supernatant (0.5 ml, 2-fold dilutions) was placed in 30 mm plastic

dishes (bacteriological grade) and overlaid with the methocel-NRK indicator cell mixture. Dishes were incubated for 10 days at 37°C and colonies were counted using an inverted light microscope. Results are presented as numbers of NRK colonies/ 10^6 transfected or control cells/3d/20 ml supernatant. Background colony numbers obtained with 1% FCS-medium have been subtracted. The data is derived from the results of six plates from three experiments.

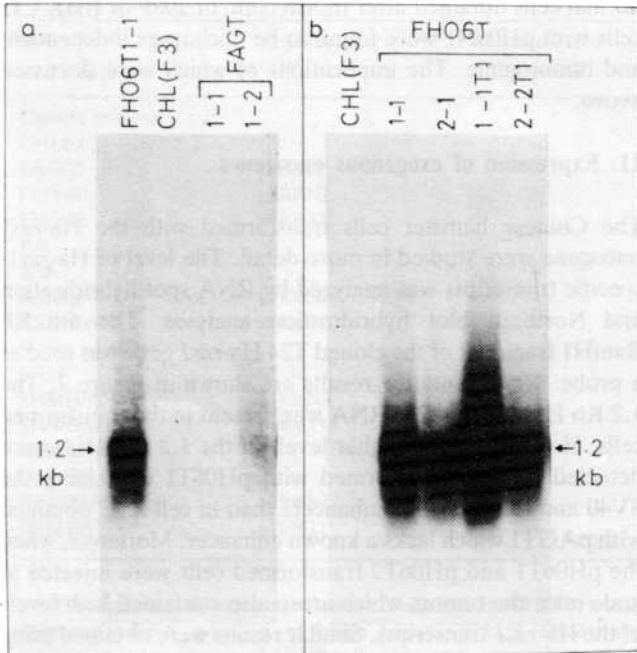


Figure 8. Autoradiographs showing Northern blot hybridization analysis of *Ha-ras1* RNA (a) in recipient CHL (F3) and transfected with pAGT1 (FAGT1-1 or FAGT1-2) or pH06T1 (FHO6T1-1) cells and (b) transformed Chinese hamster lung cells growing in culture (FHO6T1-1, FHO6T2-1) or as tumours in nude mice (FHO6T1-1T1, FHO6T2-1T1). Twenty µg of total cell RNAs from cells indicated on the Figure were fractionated on 1% agarose-formaldehyde containing gels and blotted onto nitrocellulose as previously described (103). ³²P-labelled 6.6 kb BamHI fragment carrying the T24 oncogene was used as probe.

Table VII. Transient TGF release by transfected Chinese hamster lung (CHLF3) cells^a.

Donor DNA	Exogenous oncogene	Enhancers	TGF release AV±SD
-	-	-	1.4±1.2
pAG60	-	-	1.2±0.7
pAGN1	Ha-ras1	-	1.5±1.0
pAGT1	T24 Ha-ras1	-	1.5±4.4
Homer 6	-	SV40,MoMSV	0.9±0.7
pH06N1	Ha-ras1	»	1.4±7.2
pH06T1	T24 Ha-ras1	»	55±16
Homer 5	-	SV40	1.2±0.9
pH05T1	T24 Ha-ras1	»	52±20

^aDonor DNA was added to third passage CHLF3 cells using the calcium phosphate technique (4) as modified (109). Briefly, the recipient cells were plated the day before at the concentration of 1X10⁶ cells/75 cm² flask. 2 mls the calcium phosphate DNA coprecipitate (at 20 µg DNA/ml in the absence of carrier) was added per flask containing 20 ml of growth medium (Ham's SF12 + 15% FCS). After 24 h at 37°C the medium was removed and replaced with 20 mls of low serum medium (Ham's SF12 containing 1% FCS). The medium was collected after a further 3 days incubation at 37°C, centrifuged to remove any cells the supernatant was filtered and assayed for TGFs as described in Table VI.

Table VIII. EGF-competing activity in supernatants of transfected Chinese hamster lung cells ^a.

Donor DNA	Exogenous oncogene	Enhancers	%EGF competition on NRK cells
-	-	-	9
pAG60	-	-	7
pAGN1	Ha-ras1	-	13
pAGT1	T24 Ha-ras1	-	30
Homer 6	-	SV40,MoMSV	8
pH06N1	Ha-ras1	»	49
pH06T1	T24 Ha-ras1	»	82
Homer 5	-	SV40	7
pH05T1	T24 Ha-ras1	»	77

^aThe EGF competition was determined by using NRK cells in a radioreceptor assay. Competitions were initiated by the addition of 1.0 ml of binding buffer (1x Dulbecco's modification of Eagle's medium with 3.70 g/l sodium bicarbonate and 0.1% bovine serum albumin) with or without the potential inhibitor, obtained after lyophilization of 5 ml supernatants from transfected cells, to 1X10⁵ cells plated the previous day in 16 mm diameter wells (Costar). The mixtures were incubated with the monolayer at 37°C for 1 h, the fluid was removed and the cells were washed three times with 1 ml portions of binding buffer. Binding of EGF was initiated immediately by adding 0.4 ng of ¹²⁵I-labelled EGF (Amersham) in 0.5 ml of binding buffer. After incubation for 1 h at 37°C the quantity of ¹²⁵I-labelled EGF specifically bound was determined (106). The data are derived from the results of two wells per inhibitor from two separate experiments and the average is given.

large number of natural and experimentally induced tumors (for a review, see Reference 104). The study of tumor heterogeneity is fundamentally important in order to understand the mechanism(s) of neoplasia and to obtain a better treatment of malignant disease.

We have approached this problem by studying the chromosome patterns of rodent cell lines derived after immortalization or tumorigenic conversion of early passage cells with recombinants carrying the T24 or the normal human *Ha-ras1* gene. We found that cells of different mammalian species respond differently to an introduced cloned human oncogene with respect to karyotype stability (105). We have suggested that these may reflect differences in susceptibility to cell transformation *in vitro* and carcinogenesis *in vivo* (105).

In particular, we have demonstrated that morphologically normal Chinese hamster lung cells obtained after transfection of early passage cells with recombinant plasmids carrying the T24 or a normal human *Ha-ras1* gene linked to a transcriptional enhancer have karyotypes similar to those of normal cells but with a small number of cells showing chromosome abnormalities. In comparison, fully transformed malignant cells obtained after transfection or early passage Chinese hamster cells with recombinants carrying the T24 *Ha-ras1* gene surrounded by SV40 and MoMSV LTR enhancers possessed significantly altered karyotypes. When malignant transformed rat cells were passaged in culture, progressive

changes from aneuploidy to heteroploidy were observed. However, malignantly transformed Syrian and Chinese cells maintained an aneuploid phenotype with passage (105).

We have also found karyotypic heterogeneity within the various tumorigenic cell lines obtained after malignant transformation of early passage Chinese hamster cells. Each cell line had a distinct karyotype with marker chromosomes (105). We have speculated previously that the chromosomal alterations which generate marker chromosomes may be required to fix the transformed phenotype during the late stages of transformation with these recombinant plasmids (14).

13. Transforming growth factors (TGFs) and autocrine stimulation

Transforming growth factors were initially identified in the supernatant medium of retrovirus transformed rodent cells (106). Subsequently, they were found to be produced by a variety of virus transformed cells, tumors and normal cells (for a review, see Reference 107). The autocrine stimulation hypothesis of Sporn and Todaro (36) proposed that cells could become malignant by the production of polypeptide growth factors acting on their producer cells via cell membrane receptors, allowing expression of the factor by the same cell that produces it. This hypothesis has received substantial support over the last few years. Recently growth factors have been divided into positive autocrine growth factors and negative autocrine growth factors (107). It has been suggested that malignant cell transformation could arise not only from excessive production, expression and action of positive autocrine growth factors but also from the failure of cells to synthesize, express or respond to the specific negative growth factors which they normally release to control their own growth (107).

The functional interaction between growth factors and oncogenes has been examined in some cases. Thus, stimulation of serum starved cells with peptide growth factors causes transcriptional activation of the *fos* and *myc* oncogenes (108). I describe below some of my experiments aimed to find out if TGF induction can be triggered by exogenously added oncogenes.

14. Secretion of transforming growth factors (TGFs) by cells carrying exogenous oncogenes

Cells from geneticin resistant colonies obtained after transfection with recombinants carrying oncogenes were further propagated and tested for TGF release (106). The assay involves growth of cells in medium containing low serum for 3 days then the supernatant fluid is added to NRK indicator rat cells in semisolid medium to test for TGF activity. Results from representative clones are shown in Table VI. Whereas the recipient cells alone released background amounts of TGF, all anchorage independent transformed cells secreted

large amounts. However, early passage Chinese and Syrian hamster or rat cells immortalized with the pH06N1 recombinant plasmid released intermediate TGF levels. Finally, 208F cells transformed with recombinants carrying enhancers and the HT1080 N-ras or c-myc, or v-myc genes secreted large amounts of TGF similar to that previously observed with 208F cells transformed with pAGT1, pH06T1, and pH06N1 recombinants (26). There are two main types of TGF: α and β TGF. α -TGF competes with EGF for binding to EGF receptors in a radioreceptor assay while β -TGF does not. Using this assay we have shown previously that α -TGF is produced in cells transformed by exogenous oncogenes (26).

15. Transient expression of TGF in short-term transfections of mammalian cells with oncogenes

Conditioned media from normal and transfected third passage Chinese hamster lung cells were tested for their ability to release TGF using NRK cells as the assay system. Recombinant plasmids carrying the normal or the T24 human *Ha-ras1* gene were transfected into these cells using the calcium phosphate technique (4). The release of TGFs into conditioned medium 3 days after removal of the transfecting DNA was assayed by measuring anchorage independent colonies of indicator NRK cells. The results are presented in Table VII. Several observations can be made from these data. Whereas the pAG60, Homer 6, or Homer 5 vectors alone did not induce any TGF release as compared to non-transfected cultures, recombinants carrying the T24 *Ha-ras1* oncogene in the absence (pAGT1) or in the presence (pH06T1, pH05T1), of enhancers resulted in a 10 - 30 - fold increase in TGF release. A significant, approximately 10-fold higher, TGF release than control cultures was also observed with recombinant pH06N1 carrying the normal *Ha-ras1* oncogene in the presence of enhancers. In contrast, recombinant pAGN1 carrying the same normal oncogene in the absence of enhancers failed to give any transformation. One interesting observation was that whereas recombinants pAGT1 and pH06N1 gave only about 10-fold higher TGF release than controls, recombinants pH06T1 and pH05T1 consistently gave approximately 30-fold higher TGF release.

The conditioned media from control and transfected cells were tested for their ability to compete in an EGF receptor assay. The results are shown in Table VIII. The supernatant medium from the recipient CHLF3 cells alone or from cells transfected with vector DNA or recombinant pAGN1 had little EGF competing activity. However, the conditioned medium from cells transfected with recombinants carrying the T24 *Ha-ras1* (pAGT1, pH06T1, and pH05T1) or the normal *Ha-ras1* gene in the presence of enhancers competed with the ¹²⁵I-labelled EGF for membrane receptor sites (30% for pAGT1 and 82% for pH06T1). Finally, whereas recombinants pH06T1 and pH05T1 carrying the T24 *Ha-ras1* gene in the presence of enhancers gave the highest competition values of 82% and 77% respectively, recombinants carrying

the T24 Ha-*ras1* in the absence of enhancers (pAGT1) or the normal Ha-*ras1* in the presence of enhancers (pH06N1) gave only intermediate competition values of 30% and 49% respectively.

In summary, the above experiments provide strong evidence that cloned cellular oncogenes when introduced into mammalian cells trigger induction of transforming growth factors. Since the induction occurs shortly after transfection with oncogenes this suggests that TGF release by the cell is a direct result of the oncogene action rather than a consequence of a cellular change during the transformation process.

16. Concluding Remarks

Oncogenes are mutated forms of proto-oncogenes which are genes involved in cell growth and differentiation. Both types of genes have been cloned and studied in detail. The molecular basis of their activation in some tumors has been determined. A point mutation in members of the *ras* family of oncogenes can lead to an amino acid substitution which results in the creation of a transforming p21 protein. Similarly, analyses of the expression of oncogenes in solid tumors and studies of *in vitro* transformation have indicated that elevated expression of an oncogene can also be important. Thus, both qualitative and quantitative changes play an important role in the establishment of cancer.

The discovery of transcriptional regulatory elements such as enhancers has contributed significantly to our understanding of the mechanisms involved in the carcinogenesis process. A single mutated human oncogene, the T24 Ha *ras 1* gene derived from a bladder carcinoma cell line can both trigger rescue from senescence and tumorigenic conversion of early passage rodent cells. The latter ability depends on the presence of transcriptional enhancers.

Oncogenes are capable of making cancer cells autonomous of growth factor by autocrine secretion. Thus alteration of *ras* and *myc* gene activity results in the induction of α - TGF. Therefore the evidence suggests that the various proto-oncogenes represent control signals, regulating cell growth and proliferation at different levels. The result is the existence of multiple targets where proto-oncogene alteration can give rise to malignant cell transformation. Consequently cancer can be considered as an even larger number of diseases than it was previously thought.

Oncogenes release cells from their dependence on added growth factor and confer on them growth-factor autonomy. This can occur in different ways. Our experiments suggest that oncogenes such as *ras* induce the release of particular growth factors which then may act back on the same cells which released them. Alternatively, an alteration of the trans membrane signal transducing protein could bring about the same effect.

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