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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 activiry. 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.*

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

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

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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 recepient 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 form 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 form 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

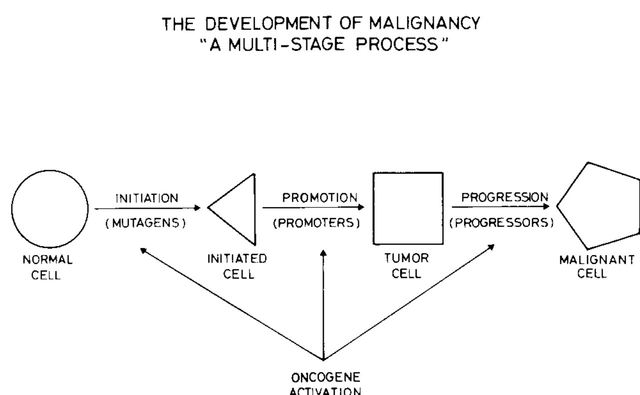

Figure 1. *The multistage nature of cancer. Substances involved in the activation of oncogenes in the initiation, promotion, and progression stages are called mutagens, promoters, and progressors, respectively.*

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 involvment 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 activitity, 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).

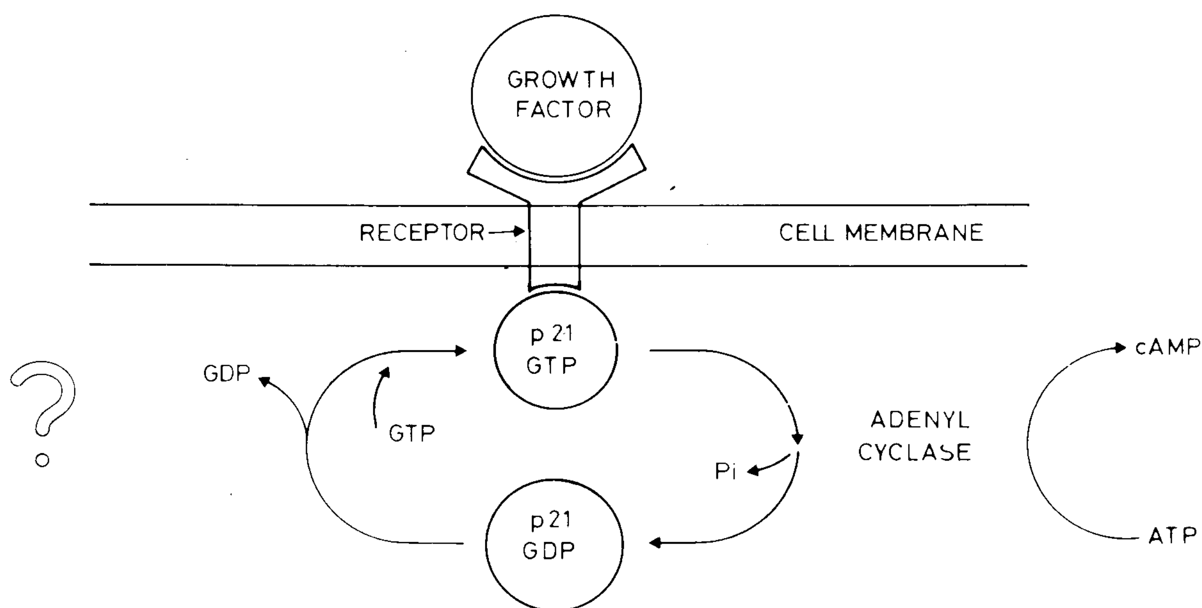

Figure 2. *Hypothetical signal transmission from growth factor to adenyl cyclase througth p21 and growth factor receptor.*

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 $\frac{AAA}{TTT}$

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 exprerimental 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

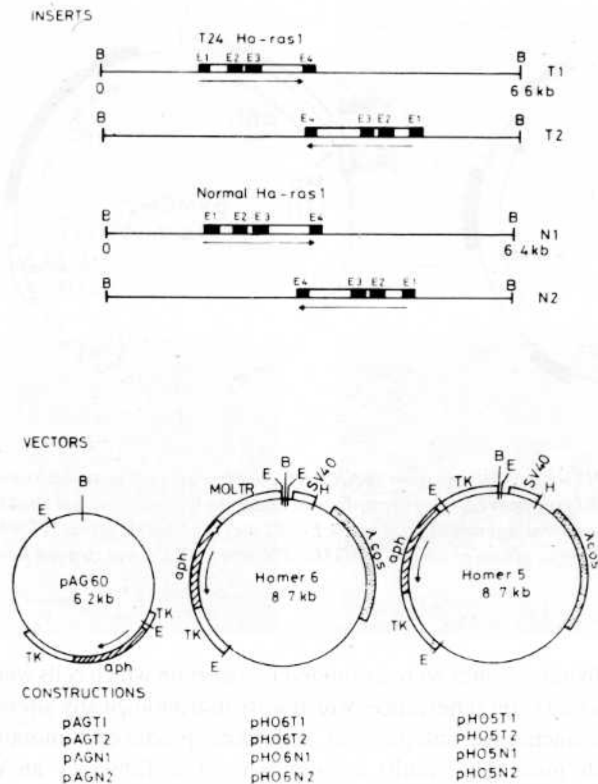

Figure 3. *Recombinant plasmids carrying the T24 Ha-ras1 oncogene (pAGT1, pAGT2, pH06T1, pH06T2, pH05T1, and pH05T2) or the normal Ha-ras1 proto-oncogene (pAGN1, pAGN2, pH06N1, pH06N2, pH05N1, and pH05N2). The 6.6 kb BamH1 fragment containing the human T24 Ha-ras1 or the 6.4 kb BamH1 fragment containing the normal human Ha-ras1 proto-oncogene were inserted into the single BamH1 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 μg 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/μg pH06T1 DNA/2×10^5 cells plated) compared to the human cells (12 colonies/μg 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-*ras*1 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/μg pH06T1 DNA/2×10^5 cells) and the lowest with 208F cells (790 colonies/μg pH06T1 DNA/2×10^5 cells). BHK C13 cells gave intermediate values for transformation frequency (5400 colonies/μg 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-*ras*1 gene (pAGT1 and pH06T1) exhibited a morhpologically transformed phenotype whereas 208F and BHK C13 cells transfected with recombinants carrying the normal Ha-*ras1* proto-oncogene (pAGN1 and pH06N1) were morhpologically 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 asssay may not always be sufficient to determine anchorage independent and tumorigenic conversion. However, when the normal Ha-*ras* gene is under the

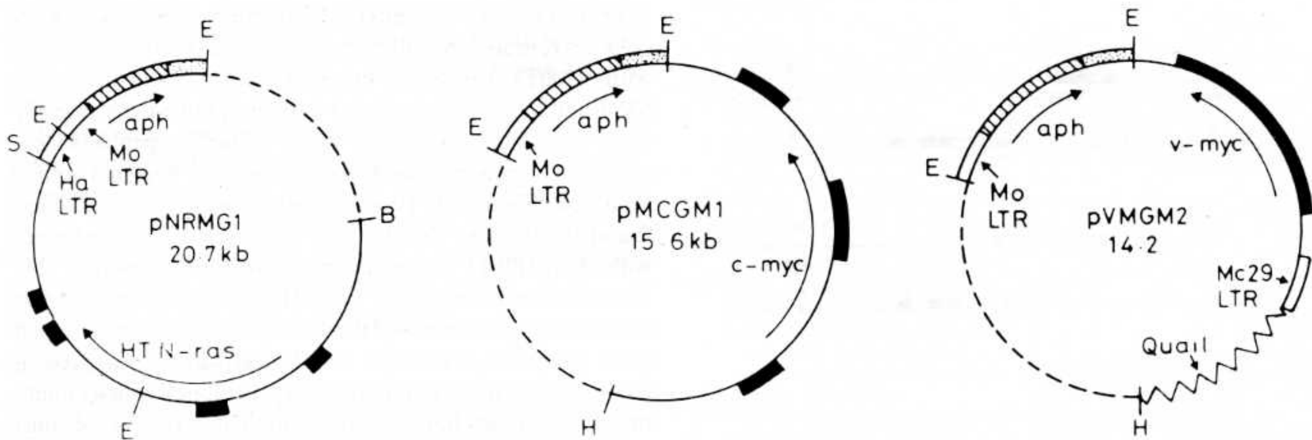

Figure 4. *Schematic representation of recombinant plasmids carrying the human HT1080 N-ras oncogene (pNRMG1), the human myc proto-oncogene (pMCGM1), and the avian retrovirus myc oncogene (pVMGM2). A 2.9 kb EcoRI fragment carrying the aph gene under the 5'transcriptional control sequences of the MoMSV 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 an 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	Genticin resistant colonies/flask or plate AV±SD	
				liquid medium	semisolid medium
SHM	pAG60	–	–	2.2± 1.4	0
	pAGN1	Ha-*rasI*	–	2.4± 1.3	0
	pAGT1	T24 Ha-*rasl*	–	2.5± 1.9	0
	Homer 6	–	SV40,MoMSV	72 ±12	0
	pH06Nl	Ha-*rasl*	»	81 ±14	0
	pH06T1	T24 Ha-*rasl*	»	81 ±12	74±15
	Salmon	–	–	0	0
GM468	pAG60	–	–	0	0
	pAGNl	Ha-*rasl*	–	0	0
	pAGTl	T24 Ha-*rasl*	–	0	0
	Homer 6	–	SV40,MoMSV	8.3± 3.0	0
	pH06Nl	Ha-*rasl*	»	10 ± 3.2	0
	pH06Tl	T24Ha-*rasl*	»	12 ± 3.5	0
	Salmon	–	–	0	0

[a]Transfection 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 cm^2 flask containing $2X10^5$ 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.

[b]The constructions of donor DNA molecules are described in Figures 3 and 4.

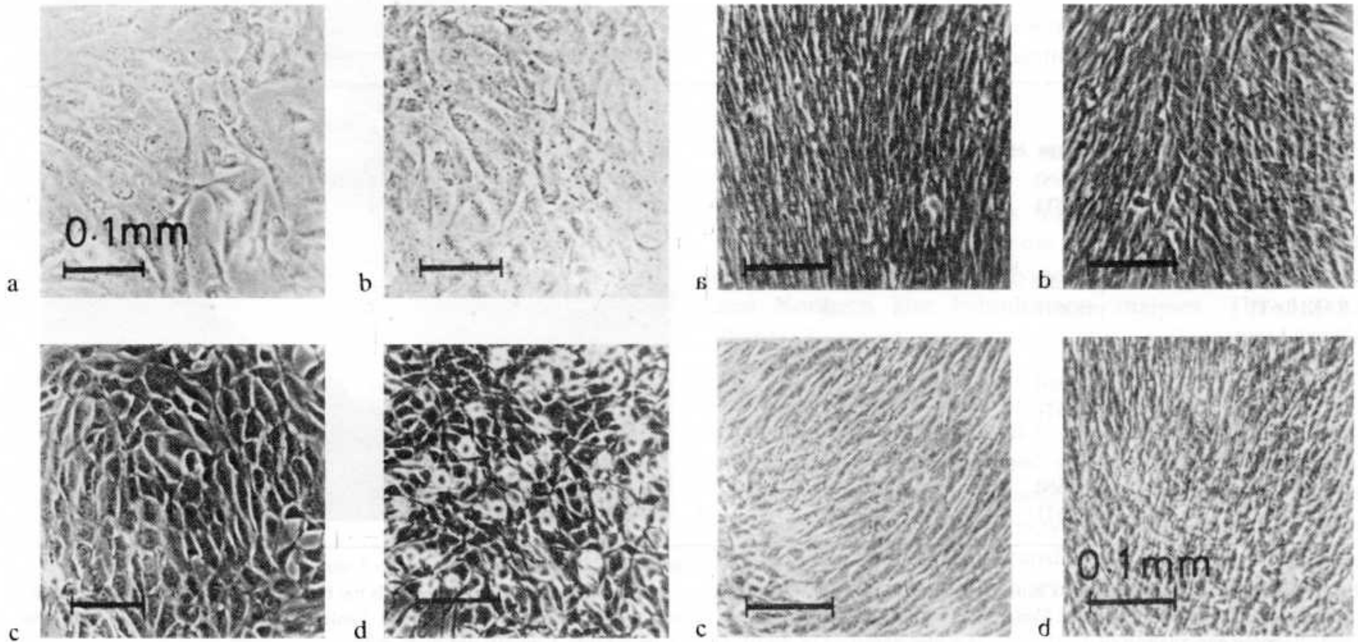

Figure 5. *Normal and transformed Syrian hamster muscle cells. (a) second passage Syrian hamster cells; (b), (c), and (d) cells transfected with Homer 6, pH06N1, and pH06T1, respectively.*

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
»	pAGNl	Ha-*ras*l	–	10 ± 3.7	0
»	pAGTl	T24Ha-*ras*l	–	11 ± 4.5	7.0± 2.1
»	Homer 6	–	SV40,MoMSV	122 ±25	0
»	pH06Nl	Ha-*ras*l	»	130 ±31	112 ±20
»	pH06Tl	T24 Ha-*ras*l	»	165 ±23	121 ±15
»	pMCGMl	c-*myc*	MoMSV	133 ±18	116 ±17
208F	pAG60	–	–	4.3± 2.7	0
»	pAGNl	Ha-*ras*l	–	4.8± 2.2	0
»	pAGTl	T24 Ha-*ras*l	–	4.7± 2.3	3.1± 1.3
»	Homer 6	–	SV40,MoMSV	76 ±15	0
»	pH06Nl	Ha-*ras*l	»	84 ±14	83 ±11
»	pH06Tl	T24 Ha-*ras*l	»	79 ± 8.8	73 ±10
»	pMCGMl	c-*myc*	MoMSV	66 ± 9.4	52 ± 6.7
BHKCl3	pAG60	–	–	2.1± 1.0	0
»	pAGNl	Ha-*ras*l	–	2.5± 1.2	0
»	pAGTl	T24 Ha-*ras*l	–	2.2± 1.2	2.0± 1.3
»	Homer 6	–	SV40,MoMSV	47 ± 7.2	0
»	pH06Nl	Ha-*ras*l	»	43 ±8.4	42 ± 7.5
»	pH06Tl	T24 Ha-*ras*l	»	54 ±10	36 ± 5.4
»	pMCGMl	c-*myc*	MoMSV	35 ± 6.4	50 ± 6.9

[a]Transfection 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.