tical significance was set at a level of $P = 0.05$.

6. The paramecium suspension was placed in a glass tube submerged in a variable temperature bath. The top of the glass tube was sealed with a rubber stopper. Two pieces of stainless steel tubing were fed through the stopper. One piece of tubing was used to bubble (about 20 ml/min) the paramecium suspension with specific gas mixtures. The other tube was a gas outlet. Samples of the paramecium suspension were withdrawn for analysis of survival with a needle pushed through the rubber stopper. Approximately 150 μl of the paramecium suspension were withdrawn from the glass tube at a time. Ten 10-μl aliquots of the suspension were placed under a microscope and the number of paramecia in each aliquot were counted to determine paramecium concentration. All paramecia examined were moving. Motionless paramecia were never observed, probably because paramecia cytolyze within minutes of dying (7).
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8. Despite a constant P_{O_2} throughout the gradient, $[O_2]$ was higher at the cold end of the gradient because O_2 solubility in water varies inversely with temperature. Thus, it is possible that the reduction in T_s with hypoxia was not due to a change in thermoregulatory behavior, but rather to a chemotaxis toward a higher ambient $[O_2]$. We think that this possibility is unlikely because (i) the chemical activity of dissolved O_2 is determined by P_{O_2}, not by $[O_2]$; (ii) diffusion of O_2 into cells is determined by P_{O_2} gradients, not by $[O_2]$ gradients; and (iii) under anoxic conditions when both P_{O_2} and $[O_2]$ were zero throughout the gradient, T_s was reduced (Fig. 1).
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Suppression of Gene Amplification in Human Cell Hybrids

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Gene amplification, one example of genetic instability, is of prognostic and clinical importance in neoplasia. In tumorigenic cells, gene amplification occurs at a very high frequency, whereas in normal diploid fibroblasts the event is undetectable by the clonogenic assay. To investigate genetic control of gene amplification, amplification frequency was measured in hybrids of tumorigenic cells and normal diploid cells. The ability to amplify an endogenous gene behaved as a recessive genetic trait, and control of gene amplification potential segregated independently of tumorigenicity and immortality.

CURRENTLY, THE DEVELOPMENT OF multiple genetic alterations is thought to be a basis of neoplasia (*1*), and increased genetic instability might be necessary to produce these changes (*2–4*). Tumorigenic cells may form from accelerated genetic changes coupled with selection pressures exerted by the surrounding microenvironment (*2*). In such a model, the accumulation of random genetic changes would ultimately lead to neoplasia. Indeed, various karyotypic changes, including aneuploidy, deletions, inversions, translocations, amplifications, and point mutations, are apparent in tumor cell populations. Such changes may contribute to the generation of cellular and biochemical heterogeneity, a hallmark of tumor cell populations (*4*).

One marker of genomic instability is gene amplification (*5, 6*), that is, an increase in the number of copies of a particular gene at a specific locus. Tumorigenic cells display a high rate of spontaneous gene amplification (*5*), as can be measured by their ability to become resistant to *N*-(phosphonoacetyl)-L-aspartate (PALA). PALA specifically inhibits the aspartate transcarbamylase activity of the multifunctional enzyme that contains carbamyl phosphate synthase, aspartate transcarbamylase, and dihydro-orotase (CAD) activities. The frequency of gene amplification–the proportion of surviving colonies in PALA relative to the number of surviving colonies without PALA—ranges from 10^{-3} in highly tumorigenic cell lines to 10^{-6} in nontumorigenic but immortalized cell lines (*5*). In contrast, gene amplification is undetectable ($<10^{-9}$) in primary, diploid cell populations (*7, 8*), reflected in the inability of normal cells to make drug-resistant clones.

We used somatic cell hybrids of cells that have a high frequency of amplification and those that have no detectable frequency of amplification to determine if the ability to amplify is a dominant or recessive trait (Table 1) (*9–13*). Such hybrids were suppressed in their ability to amplify the gene encoding the CAD enzyme. By measuring PALA resistance we analyzed both parental and hybrid cells for their ability to amplify the endogenous CAD gene. Every PALA-resistant subclone examined to date, both in rodent (*5, 14, 15*) and in human (*16*) cells, carried additional CAD gene copies and arose by amplification of the CAD locus (*17*). Although normal diploid fibroblasts showed no detectable gene amplification (*7*), highly tumorigenic fibrosarcoma and carcinoma cell lines amplified the CAD gene at high frequencies (Table 1). The SFTH400 hybrid cells, a fusion of a normal fibroblast and a fibrosarcoma cell line, displayed an amplification frequency several orders of magnitude lower than that measured in the tumorigenic parental cell line, HT1080 (Fig. 1 and Table 1). Similar results were seen with other nontumorigenic hybrid cell lines, SFTH300(S) and ESH5(S) (Table 1).

The expression or suppression of the ability to amplify was independent of the tumorigenic phenotype of the hybrid. Fusion of a normal (nonneoplastic) cell population with a highly tumorigenic cell line results in hybrid progeny with the malignant phenotype suppressed (*9*). As the hybrid cell line is propagated, however, chromosomes are lost, and rare tumorigenic segregants appear, which suggest that a specific chromosome involved in the suppression of malignancy is lost when the malignant phenotype reappears (*9–11, 18*). Reintroduction of the lost chromosome into the tumorigenic cell can completely suppress tumorigenicity (*19*). By comparing the amplification potential in suppressed hybrids and their tumorigenic segregants, we determined that suppression of gene amplification was not coupled to suppression of tumorigenicity (Fig. 1). Both SFTH400(S) cells and their

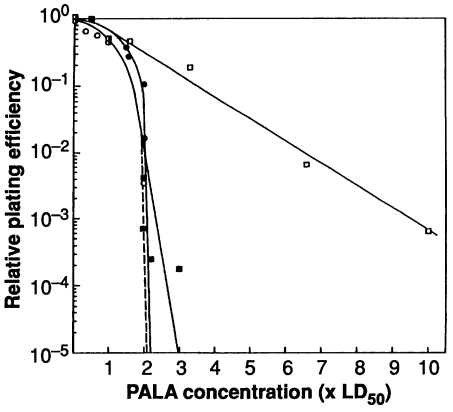

Fig. 1. Selection of somatic cell hybrids for PALA resistance. Cells were propagated and selected as in (*5*). They were plated at appropriate densities and placed in the indicated concentrations of PALA. When colony size exceeded 50 cells, plates were fixed, stained, and counted (*5*). Relative plating efficiency is the ratio of cells surviving in PALA to cells surviving in the absence of it. Amplification potential was determined at 9 × LD_{50}. Each curve is the average of a minimum of four separate determinations. Parents: open circles, GM2291 (LD_{50} = 1.5 μM); open squares, HT1080 (LD_{50} = 6 μM). Hybrids: closed circles, SFTH400(S) (LD_{50} = 10 μM); closed squares, SFTH400(T) (LD_{50} = 20 μM). Dashed line represents curve for GM2291.

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tumorigenic segregant SFTH400(T) failed to generate PALA-resistant progeny at 9 × median lethal dose (LD_{50}). The tumorigenicity of the segregant was verified by injection into nude mice; eight out of eight sites produced tumors in less than 1 week.

Normal fibroblasts appeared to contain at least one gene for suppression of gene amplification whose subsequent loss permitted reexpression of amplification potential. As previously reported, the suppressed hybrid SFTH400(S) is very stable and loses few chromosomes during fusion as well as during the generation of its tumorigenic segregant, SFTH400(T); mean chromosome number drops from 89 to between 88 and 85 (*11*) (Table 1). These cell lines retain the majority of their chromosome complement; in our study, they remained suppressed for amplification potential. In contrast, another set of hybrids between a fibroblast and fibrosarcoma also loses few chromosomes upon fusion but loses 9 different chromosomes (13 total) in generating its tumorigenic segregant (*11*; Table 1). When the segregant cells were assayed for their ability to generate PALA-resistant colonies, they reexpressed the ability to amplify the CAD locus (Table 1). Thus, these cell lines that have lost a significant component of their chromosome complement are no longer suppressed for their ability to amplify (Table 1).

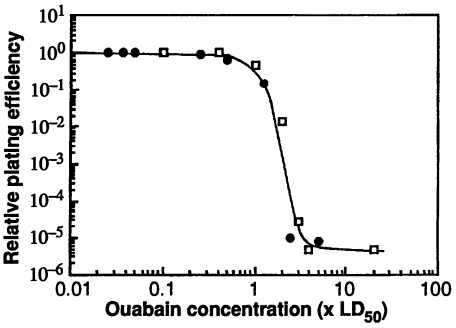

Fig. 2. Determination of ouabain resistance in parental HT1080 and its hybrid, SFTH400(S). The cells were plated at densities ranging from 10^2 to 10^6 cells per plate and placed in the indicated concentrations of ouabain. Media were changed weekly. Frequency was calculated as in (*5*). Closed circles, SFTH400(S) (LD_{50} = 2 × 10^{-6} M); open squares, HT1080 (LD_{50} = 2.5 × 10^{-8} M).

To test if two types of genomic instability—point mutations and gene amplification—are independently regulated, we used ouabain resistance as a phenotypic marker for measuring the rate of point mutations. Such resistance is the result of point mutations that lower the affinity of the mutant Na^+- and K^+-dependent adenosine triphosphate (Na^+/K^+ ATPase) enzyme for ouabain (*20*) and can be inherited in a codominant fashion. The parental HT1080 fibrosarcoma (with a high amplification potential) and the SFTH400(S) hybrid cells (with a low amplification potential) were incubated in ouabain and monitored for the appearance of drug-resistant colonies. The frequency of point mutation at this locus ($\sim 10^{-6}$) was similar in both (Fig. 2), showing that regulation of point mutations and gene amplification is independent in these cells.

Two other recessive transformation phenotypes, malignancy and immortality, have been identified through analysis of somatic cell hybrids (*21, 22*). Four complementation groups have been identified for the control of immortality (*21*), and at least four distinct and nonoverlapping complementation groups control malignancy (*22*). In our study, the continued suppression of amplification ability in hybrids that are immortal and that have reexpressed their tumorigenic potential [such as the SFTH400(T) cell line] shows that the control of gene amplification was independent of the control of malignancy and immortality in these hybrids.

The modulation of gene amplification is the third recessive phenotype associated with transformation identified by the use of human somatic cell hybrids. This phenotype is not tissue-specific, because cells of both mesenchymal and epithelial origin with the ability to amplify were suppressed by diploid mesenchymal cells (fibroblasts) (Table 1). Overall, these results demonstrate that in these particular hybrids the ability to amplify is recessive but do not preclude the possibility that dominant genes may also exist that modulate the frequency of gene amplification.

The initiation and progression of tumorigenesis is a multistep process that results in the deregulation of many basic cellular functions such as proliferation, adhesion, cell motility, immortality, and proteolytic activity (*23*). Our data hold implications for the role of genomic instability in this process. If the role of genomic instability is to generate the transforming mutations that lead to tumorigenesis, then once the requisite mutations are accomplished genomic instability would no longer be necessary for the expressed tumorigenicity of the cell, and the genetic determinants of each process would be expected to be independent. If, on the other hand, genomic instability (as measured here by gene amplification) is a phenotypic consequence of the tumor state, then the two properties should cosegregate and all tumorigenic segregants would be expected to demonstrate amplification ability. Our separation of these two processes demonstrates that the ability to amplify (per se) is not a phenotypic consequence of tumorigenicity and that tumorigenicity and amplification ability are under independent control. These results are consistent with the interpretation that increased genomic instability underlies the generation of genetic alterations that accumulate and occasionally terminate in neoplasia. A basic difference between normal, diploid cells and neoplastic cells may be the frequency with which genetically altered sites are generated or accumulate. Our results suggest that genomic instability is suppressed in normal diploid cells and that the loss of this suppression could underlie the generation of the genotypic changes noted in multistep carcinogenesis (*1, 24*).

Table 1. Characteristics of parental and hybrid human cells; n.a., no longer available; (S), suppressed for tumorigenicity; (T), tumorigenic.

Cell line	Description	Chromosomes (*n*)	Amplification frequency	Tumorigenicity*	Cell line reference
		Parental cells			
GM2291†	Lung fibroblast	46	$<10^{-9}$	0/6	(*11, 12*)
$75\text{–}18^{OR}$	Fibroblast	46	n.a.	0/6	(*11, 12*)
GM0077	Fibroblast	46	$<10^{-8}$	0/10	(*11, 25*)
HT1080	Fibrosarcoma	46	10^{-3}	6/6	(*11*)
D98/AH-2	Cervical carcinoma (HeLa cell line)	61	10^{-4}	6/6	(*9, 12, 22*)
		Hybrid cells			
SFTH400(S)	$75\text{–}18^{OR}$ × HT1080‡	89	$<2 \times 10^{-7}$	0/6	(*11*)
SFTH400(T)	$75\text{–}18^{OR}$ × HT1080	85–88	$<10^{-8}$	6/6	(*11*)
SFTH300(S)	GM2291 × HT1080§	88	$<10^{-7}$	0/6	(*11*)
SFTH300(T)‖	GM2291 × HT1080	75	1.4×10^{-4}	6/6	(*11*)
ESH5(S)	GM0077 × D98/AH-2	102	$<10^{-7}$	0/6	(*9, 25*)

*Data as described (*9, 11*). †Ouabain-resistant derivative. ‡Derivative of HT1080 (HTD114Cl) defective in adenosine phosphoribosyl transferase gene. §Derivative of HT1080 (6TG C5) resistant to 6-thioguanine. ‖Subclone SFTH300V-T1.

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13. Diploid fibroblasts were hybridized with two tumorigenic cell lines of different tissue origin, a fibrosarcoma and a carcinoma. The progeny were extremely stable with a minimal loss of chromosomes after fusion (*9, 11*). The tumorigenicity of these hybrids was initially suppressed but, upon chromosome loss, was reexpressed (*11*). We verified that these hybrids still contained the expected chromosome complement and proliferated in the indicated selective media [hypoxanthine, aminopterin, thymidine (HAT) plus 5×10^{-7} M ouabain or 5×10^{-5} M ouabain alone (*11*)]. Both tumorigenic and nontumorigenic hybrids grew at rates comparable to the parental cell lines.
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Formation of Ion-Permeable Channels by Tumor Necrosis Factor–α

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Tumor necrosis factor–α (TNF, cachectin), a protein secreted by activated macrophages, participates in inflammatory responses and in infectious and neoplastic disease states. The mechanisms by which TNF exerts cytotoxic, hormonal, and other specific effects are obscure. Structural studies of the TNF trimer have revealed a central pore-like region. Although several amino acid side chains appear to preclude an open channel, the ability of TNF to insert into lipid vesicles raised the possibility that opening might occur in a bilayer milieu. Acidification of TNF promoted conformational changes concordant with increased surface hydrophobicity and membrane insertion. Furthermore, TNF formed pH-dependent, voltage-dependent, ion-permeable channels in planar lipid bilayer membranes and increased the sodium permeability of human U937 histiocytic lymphoma cells. Thus, some of the physiological effects of TNF may be elicited through its intrinsic ion channel–forming activity.

THE PROTEIN TNF CAUSES HEMORrhagic necrosis of certain solid tumors and is responsible for the physiological manifestations of "endotoxic shock" and the wasting syndrome (cachexia) of chronic disease. It also participates in positive and negative growth regulation and has direct effects on a wide variety of cells (*1*). Although TNF typically acts to combat infection and cancer growth, it notably enhances human immunodeficiency virus replication (*2*) and has permeabilizing effects on the vascular endothelium that may actually contribute to cancer metastasis (*3*).

Mature TNF acts as a compact trimer (~51 kD); each subunit is an antiparallel β barrel (*4, 5*). A long channel extends down the central axis of threefold symmetry (*4, 5*). Despite the compactness of the trimeric structure and the occluded appearance of the midregion of the channel, the trimer interface may be somewhat plastic because the three monomeric subunits are not precisely superimposable (*5*). The ability of TNF to insert into membranes (*6*) and to dissociate in mild detergent (*7*) supports this concept of interface plasticity. TNF insertion into the hydrocarbon core of phospholipid bilayers increases with decreasing pH (*6*). Acidification (pH 5.3, 15 min) also increases the cancer cell–killing activity of TNF (*6, 8*) while reducing specific binding (*9*). Two studies with model membrane targets have shown that TNF can increase vesicle permeability to calcein (*10*). Moreover, several physiological studies suggest TNF alters membrane permeability. Thus, TNF causes a rapid decrease in the resting membrane potential of skeletal muscle cells (*11*), similar to that observed in the endotoxic shock syndrome (*12*); this activity could account for the increased sodium space and fluid retention that occurs in shock. TNF also induces oligodendrocyte necrosis, myelin dilatation, and periaxonal swelling similar to that induced by batrachotoxin (which opens Na^+ channels) (*13*). To determine whether TNF might itself be a channel-forming molecule, a hypothesis consonant with its channel-like structure and membrane insertion activity, we evaluated the effects of human recombinant TNF on planar phospholipid membranes.

Figure 1A shows the current response of a TNF-treated membrane to a series of graded voltage (v) steps. In the absence of TNF, the membrane conductance was ohmic and equal to ~10 pS (indistinguishable from zero in Fig. 1). After addition of TNF to a final concentration of 100 ng/ml, the membrane current I (and therefore conductance, $g = I/V$) remained nearly zero at small voltages (absolute values of $V < 40$ mV). The side to which TNF was added was taken as ground; hence, voltages correspond to the "cytoplasmic" voltage. At larger positive voltages ($V > 40$ mV), the current rose within seconds to a new, higher steady state, which was steeply dependent on the membrane voltage. When the polarity of the voltage was reversed, the conductance de-

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