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SOMATIC CELL GENETICS AND ITS APPLICATION TO MEDICINE

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INTRODUCTION

Somatic cell genetics has become a new branch of genetics, affording understanding of gene and chromosome structure and their activities in multicellular eukaryotic organisms generally and man in particular. Genetic analysis by examination of somatic cells in vitro bypasses the need for human mating, greatly accelerates the possible rate of experimentation, and readily lends itself to molecular biological approaches. In addition, it has introduced fundamental new approaches to medicine. It has become the principal, though of course not the only, means for study of human genetics today. This paper briefly reviews the history of this approach, indicates the powers that it makes available for exploration of genetic processes in vitro, and examines their application to problems of medicine. [For a detailed review of this subject before 1972, see (222). For other more recent reviews dealing with certain aspects of somatic cell genetics, see (42, 162, 238, 240, 241).]

With the discovery in 1953 of the three-dimensional structure of DNA, the era of molecular biology was born (287). This development was a fusion of the hitherto independent disciplines of genetics and biochemistry and was first applied to the simplest living cells like *E. coli.* In attempting similarly to construct a molecular biology of man, a formidable obstacle became obvious. While study of human biochemistry possessed all of the powers of biochemical studies on other organisms, the powers of human genetics in 1953 were greatly limited for two reasons. In contrast to the generation time of 20 minutes for *E. coli,* that of man approximates 25 years. Moreover, it is not possible in man to carry out those specific matings that would provide the needed answers to particular genetic questions.

A solution to this problem was offered in 1955 by Puck & Marcus (227, 228). Since the human body contains approximately 10^{13} nucleated somatic cells, it was proposed to treat these as though they were equivalent to *E. coli* cells and apply the methods of microbial genetics to such cell populations. Thus study of the genetics of somatic cells would be substituted for that of the germ cells utilized in classical eukaryotic genetics.

This review, which cannot be encyclopedic in scope, treats a set of arbitrarily selected topics of somatic cell genetics and has drawn most heavily

on historical developments that have taken place in the laboratories of the Eleanor Roosevelt Institute for Cancer Research and the Lita A. Hazen Laboratories for the Study of Genetic and Developmental Medicine, since these afford illustrative examples with the least labor on the part of the authors.

THE BASIC METHODOLOGIES

The techniques required in order to accomplish genetic analysis of somatic cells are as follows:

1. To develop a simple, rapid and reliable means for growing single cells into clonal populations, permitting selection, recognition, and isolation of mutant forms, just as is done with *E. coli.*
2. To develop a method for simple and reliable sampling of somatic cells from any person or experimental animal, and their establishment in long-term cultures.
3. To develop the necessary technology for growth of such cell cultures into large and reasonably stable populations without the chromosomal disorganization which had characterized tissue culture cell lines in the past.

In the course of these developments, it was necessary to design incubators that would precisely control temperature, relative humidity, and CO_2 concentration; to formulate media and conditions conducive to reliable growth of single cells into colonies with high efficiency; and to design methods for isolating colonies, growing new stocks, and insuring their clonal nature.

By 1958, all of these steps had been accomplished (87, 223, 227, 229) and somatic cell genetics was launched as a new approach to the genetics of man and other eukaryotic multicellular organisms. This approach has provided the power to elucidate the structure and functions of the genomes of somatic cells of eukaryotes, and has produced a combination of in vivo and in vitro studies that has illuminated a wide variety of genetic problems.

SINGLE CELL SURVIVAL CURVES

The precision with which growth of single mammalian cells into colonies can be achieved made possible construction of single cell survival curves that quantitatively measure the effects of various agents and procedures on the reproductive capacity of somatic mammalian cells. The procedure involves depositing measured aliquots of cells in a series of petri dishes; exposing these to successively larger doses of the agent whose effect is to be measured; incubating all the plates; and counting the developing colonies containing 50 or more cells. Thus, for the first time, it became possible to

obtain meaningful quantitative measurements of the mean lethal dose of physical, chemical, and biological agents on mammalian cell reproduction (186, 214, 215, 224, 226, 228, 230). Such curves have proved useful in isolating and characterizing mutants (128, 129), in the analysis of chromosomal damage (129, 214, 215), in study of genetic repair processes (285), in the analysis of the action of viruses and drugs (129, 159), and in the immunogenetics of antibodies to cell surface antigens (186, 232). They have been particularly useful in mammalian radiobiology where they have made possible new quantitative understanding of radiation processes in mammals at levels ranging from single cells to the whole organism (see section on mammalian radiobiology and human radiotherapy).

THE HUMAN CHROMOSOMES AND THE CHROMOSOMAL DISEASES

The first level at which eukaryotic genetic analysis must begin is that of the chromosomes. When somatic cell genetics was first introduced, not even the number of human chromosomes was known. Following the introduction of the hypotonic method for expanding cells (98), which separated the chromosomes from each other, Tjio & Levan (278) first determined that the correct value for the human cell chromosome number was 46 instead of 48, an erroneous value which had been accepted by geneticists for more than 25 years.

The first complete identification of the human chromosomes was published by Tjio & Puck in 1958 (279, 280). Soon afterwards, however, several alternative classification systems of the human chromosomes were proposed, and a conference was assembled in Denver to produce a new classification system that could be universally adopted. This self-styled Human Chromosome Study Group consisted of 13 geneticists who had already published provisional somatic human karyotypes, and three other distinguished geneticists to act as counselors and arbiters of any disagreements which might arise. The group produced a document describing the Denver Classification of the Human Chromosomes (52), which has since become the system in universal use. This document has been republished in scores of different journals and in many languages. It was a matter of great gratification to the participants in that conference that decades later, when the chromosomal banding techniques, which provided much higher resolving power for each of the human chromosomes, were introduced, not one of the classifications of the Denver System needed to be changed. Additional international conferences on the human chromosomes were subsequently held in London, Chicago, and Paris, and helped to define further details of the human karyotype in health and disease (30, 154, 199).

When chromosome banding techniques were first introduced by Caspersson (26), enormous improvement was effected in the ease of recognizing particular chromosomes and identifying their component parts. A variety of banding techniques are now available as routine operations (199). Together with the new methodologies introduced by Yunis (299) and Francke & Oliver (65) utilizing the expanded length of the late prophase and prometaphase chromosomes, it is now possible to increase greatly the resolving power of human somatic chromosome examination over that which was available when the Denver Classification System was first adopted.

Another important advance in chromosomal characterization has been the use of in situ hybridization first introduced by Pardue & Gall (198). It permits, by means of radioautography or other visualization techniques, such as fluorescence, (140), identification of specific positions at which particular nucleotide sequences can attach to the mitotic chromosomes. These approaches are considered further in subsequent sections of this paper dealing with human gene mapping.

The demonstration by Lejeune (149) that Down syndrome is due to trisomy 21 was a revolutionary advance leading to a new field of medicine. Medical cytogenetics has now demonstrated that at least 60 or 70 human diseases, some of them having existed as medical enigmas for many decades, are the result of aneuploidy of partial or total chromosomal members readily identified under the microscope. The enormous growth of knowledge in this field has been summarized in several recent reviews (13, 80, 150). The definition of specific chromosomal anomalies involved in particular syndromes is permitting steadily increasing powers of pre- as well as post-natal diagnosis, and will, in addition, illuminate specific genome involvement in normal human developmental processes.

MAMMALIAN RADIOBIOLOGY AND HUMAN RADIOTHERAPY

One of the first applications of somatic cell genetics occurred in the field of radiobiology and the radiotherapy of tumors. The previously generally accepted value for the mean lethal dose of ionizing radiation for mammalian cells lay in the region between 50,000 and 100,000 rads. Since it was known that the lethal dose of total body radiation for most mammals lay in the neighborhood of 300–400 rads, destruction of the ability of cells to reproduce was considered to play a relatively minor role in the mammalian radiation syndrome. Moreover, there was no generally accepted conceptual theory to guide the selection of doses to be applied to tumors in radiotherapy. As a consequence, the specific effects of irradiation on mammalian cells

and body tissues presented confusing situations for the radiobiologist and the radiotherapist.

Important clarification resulted when the single cell survival curve for X-irradiation of mammalian cells was determined (228, 230). These data demonstrated that the mean lethal dose of ionizing radiation for mammalian cells was only 100 rads instead of the vastly greater quantity that had been earlier conceived. The value was relatively constant for most mammals and, indeed, was very similar for normal cells and cancer cells, thus ending the earlier supposition that cancer radiotherapy was successful because of the greater susceptibility of the malignant cell. The need, therefore, for careful delivery of the dose specifically to the malignant cells was emphasized.

A variety of considerations and experimental findings were presented to show that the process responsible for reproductive death of the cells was chromosomal damage consisting mainly of breaks and their consequences (214). The initial shoulder in the single cell survival curve was demonstrated to be due to normal cell repair processes, as evidenced by the fact that caffeine, which inhibits repair, causes HeLa cells to exhibit a survival curve with no initial shoulder, but with the same final slope as that obtained in the absence of this agent (285). Finally, the human genetic disease, Xeroderma Pigmentosum, was shown to be due to the presence of any of several different genetic defects, each of which results in failure of normal repair processes (12, 34). Thus the greater susceptibility of such persons to radiation damage in general and radiation-induced cancer in particular became clear. These developments illustrate how obscure areas in medicine have been illuminated as a result of application of somatic cell genetic methodologies in quantitative fashion.

It also became possible to shed light on mysteries surrounding the nature of a large part of the pathology underlying the mammalian radiation syndrome. For example, it had been known for decades that certain tissues like the bone marrow display profound pathology including cell depletion following irradiation with relatively small doses of ionizing radiation in the neighborhood of 150–250 rads, while other tissues like muscle fail to display significant change even after exposure to thousands of rads (218). While a variety of different speculations had been offered to account for these tissue-specific susceptibilities, none of them could be substantiated.

Experimental demonstration that these differential degrees of tissue cell depletion were due to intrinsic differences in cell turnover in the various normal mammalian tissues was first produced by use of the approaches of somatic cell genetics (216–218). Measurements of the rate of cell depletion as a function of radiation dose and time were carried out in a variety of tissues of the mouse, and the data were analyzed in relation to the single

cell survival curve (219). Such experiments demonstrated that the dose at which cell depletion is first observed and the limiting dose beyond which no further increase in cell depletion occurs obey relationships predicted from the appropriate single cell survival curves. Tissues with a high rate of cell turnover are rapidly depleted when irradiated with doses which destroy most of the reproductive capacity of their cells. On the other hand, cells like muscle, which normally exhibit little or no turnover, exhibit little or no cell depletion even after absorption of large doses of ionizing radiation. Indeed, it was shown that the cell depletion curve obtained from exposure of a particular tissue to a series of different radiation doses in successive experiments could be used to measure the normal turnover time of the cells in that tissue (216, 217, 219).

SINGLE GENE MUTATIONS

General Considerations

The first in vitro induction of single gene mutations by mutagenic action in mammalian cells was described by Kao & Puck (128) in an approach designed to produce and isolate auxotrophic mutants. In the same year, Chu & Malling (32) produced drug-resistant mutants using the direct selection procedure introduced by Szybalski. Later other types of mutants were produced, including temperature-sensitive mutants (276), other auxotrophs, and mutants resistant to a variety of drugs (for review see 256). Mutants have been isolated in diploid human cells (50) as well as in cells from a variety of experimental animals.

For distinguishing recessive point mutations from stable phenotypic changes and from more complex genetic changes, the following operational criteria have been proposed (134): (*a*) the spontaneous frequencies of forward and reverse mutations are low, and can be significantly increased by mutagenesis; (*b*) when hybridized with appropriate cells, the mutant displays recessive characteristics and a pattern of complementing behavior consistent with that of a single gene mutation; (*c*) a complementing human or other mammalian chromosome can be identified; and (*d*) alteration or deficiency of a specific protein or enzyme is demonstrable. As more mammalian somatic cell variants resulting from treatment with mutagenic agents have been isolated and characterized, abundant evidence has accumulated demonstrating underlying genetic, rather than epigenetic, alterations (50, 256). With the development of gene mapping techniques with ever-increasing resolving power, it seems likely that specific point mutations will soon be generally characterized in terms of their corresponding changes in specific nucleotide sequences.

Auxotrophic Mutants of the Chinese Hamster Ovary Cell

The successful isolation of a large variety of auxotrophic mutants in Chinese hamster ovary (CHO) cells has facilitated many kinds of experimental approaches (222). Table 1 presents a list of auxotrophic mutants isolated in the CHO and other Chinese hamster cell lines. The CHO cell was established in 1958 after spontaneous transformation of a Chinese hamster ovary cell culture (280), and has been in continuous culture ever since. A subclone, designated CHO-K1, was selected on the basis of its modal chromosome number being 20, instead of 21 as in the original CHO cell (128).

The CHO-K1 cell has proved to be unusually versatile and convenient for genetic experiments. It can be grown with 100% plating efficiency either attached to surfaces or in suspension. The generation time of approximately 11 hours makes possible rapid growth of large cell populations. It has a small modal chromosome number of 20 and a relatively stable chromosomal constitution, although a number of alterations from the normal karyotype are present. It readily hybridizes with other cells, and its hybrids with human cells rapidly eject unnecessary human chromosomes, so that when appropriate selective markers are available, specific human chromosomes can be retained in a stable hybrid. Cell surface antigens are readily distinguished from those of the human, so that convenient and selective immunological markers can readily be prepared. The CHO cell can easily be mutagenized by a large variety of mutagens providing many kinds of markers that have been extremely useful in genetic analysis, often with a high degree of resolving power.

For explaining the high frequency of recessive mutants occurring in CHO-K1 and other cells, Siminovitch (256) postulated a mechanism in which many genes in this cell are functionally hemizygous, although two copies of each gene are still present. This hemizygosity could have resulted from the extensive chromosomal rearrangements that have occurred in CHO cells (45, 129). Some lines of evidence supporting the functional hemizygosity hypothesis have been described in CHO cells (23, 81–83). However, the generality of this proposed mechanism remains to be established. Certainly not all genes appear to be functionally haploid in CHO cells (29, 118, 255).

Chromosomal assignment of typical human genes complementing mutant enzyme deficiencies have been achieved for the following mutants listed in Table I: in CHO-K1/pro$^-$ cells: pro$^-$ to human chromosome 10 (107), gly$^-$A to chromosome 12 (116), ade$^-$A to chromosome 4 (267), ade$^-$C to chromosome 21 (179), ade$^-$E to chromosome 14 (113), ade$^-$B to chromosome 14 (121), ade$^-$G to chromosome 21 (206), GAT$^-$ to chromosome 9 (109).

Table 1 Auxotrophic mutants in CHO and other Chinese hamster cells[a]

Parent	Mutant	Nutritional requirement	Enzyme defect	References
CHO	pro$^-$	Proline or Δ^1-pyrroline-5-carboxylic acid	Defective in converting glutamic acid to glutamic γ-semialdehyde	86, 107, 127, 133
CHO-K1/pro$^-$	gly$^-$A	Glycine	Serine hydroxymethyltransferase	29, 116, 122, 142
	gly$^-$B	Glycine or folinic acid		122
	gly$^-$C	Glycine		122
	gly$^-$D	Glycine		122
	ade$^-$A	Adenine, hypoxanthine, their ribonucleosides, or ribonucleotides, or 5-aminoimidazole-4-carboxamide	Amidophosphoribosyltransferase	131, 200, 205
	ade$^-$B	Like ade$^-$A	Phosphoribosylformylglycinamidine synthetase	121, 131, 132, 200, 205
	ade$^-$C	Like ade$^-$A	Phosphoribosylglycinamide synthetase	179, 200, 205
	ade$^-$D	Like ade$^-$A	Phosphoribosyl-amino imidazole carboxylase	200, 205
	ade$^-$E	Like ade$^-$A	Phosphoribosylglycinamide formyltransferase	113, 200, 205
	ade$^-$F	Like ade$^-$A, except unable to utilize 5-aminoimidazole-4-carboxamide	Phosphoribosylaminoimidazole carboxamide formyltransferase	200, 205
	ade$^-$G	Like ade$^-$A	Phosphoribosylaminoimidazole synthetase	206
	ade$^-$H	Adenine	Adenylosuccinate synthetase	202, 282
	ade$^-$I	Adenine	Adenylosuccinate lyase	202
	ade$^-$P$_{AB}$	Like ade$^-$A	Amidophosphoribosyltransferase and phosphoribosylformylglycinamidine synthetase, not complementating either ade$^-$A or ade$^-$B	185
	Urd$^-$A	Uridine, orotic acid or dihydroorotic acid	Carbamyl phosphate synthetase, aspartate transcarbamylase, and dihydroorotase	40, 41, 204
	Urd$^-$B	Slower growth, not requiring uridine	Dihydroorotate dehydrogenase	265
	Urd$^-$C	Uridine	Orotate phosphoribosyltransferase and OMP decarboxylase	203
	trans$^-$	Unable to synthesize valine, leucine or isoleucine from its respective α-keto acid	Branched-chain amino acid transaminase	110
	ala$^-$	Alanine	Alanyl-tRNA synthetase	88
	glu$^-$	Glutamate		88
	ino$^-$	Inositol		130, 133, 134
	GAT$^-$	Glycine-adenine-thymidine	Folylpolyglutamate synthetase	109, 128, 131
	pur$^-$	Like ade$^-$A	Phosphoribosylformylgycinamide synthetase	57, 272
	tsGAT$^-$	Glycine-adenine-thymidine; auxotrophic at 39.5°C, prototrophic at 34°C		250
	Mutant #49	Unsaturated fatty acid	Microsomal stearoly-CoA desaturase	28
	Mutant #215	Cholesterol	Defective in lanosterol demethylation	27

Table 1 *(Continued)*

Parent	Mutant	Nutritional requirement	Enzyme defect	References
CHO-K1/ pro⁻gly⁻A	ser⁻	Serine		114
CHO/pro⁻	AUXB1/ GAT⁻	Glycine-adenosine-thymidine	Folylpolyglutamate synthetase	163, 273, 274
	AUXB3/ GA⁻	Glycine-adenosine	Defective in folate metabolism; not complementing AUXB1/GAT⁻	164
CHO/pro⁻ AUXB1/GAT⁻	tsAUXB1/ GAT⁻	Glycine-adenosine-thymidine; Auxotrophic at 38.5°C, prototrophic at 34°C	Defective in folate metabolism at nonpermissive temperature	
CHO/pro⁻	TSH1/leu⁻	Auxotrophic for leucine at 39.5°C, prototrophic at 34°C	Defective in leucyl-tRNA synthetase at 39.5°C	56, 178, 275, 277
	PSV3/asn	Auxotrophic for asparagine at 39.5°C, prototrophic at 34°C	Defective in aspara gyl-tRNA synthetase at 39.5°C	277
	arg⁻	Inability to utilize citrulline in place of arginine	Argininosuccinate synthetase or agininosuccinase or both	181
	cys	Inability to utilize cystathionine in place of cystine	Cystathionase	181
	Polyamine⁻	Polyamine		210
	Inosine⁻	Unable to grow on inosine as the only carbon source	Purine nucleoside phosphorylase	93
	TdR⁻	Thymidine, deoxycytidine or deoxyuridine	Inability to reduce UDP to dUDP	173
CHL	AT⁻	Adenine-thymidine	Defective in tetrahydrofolate metabolism; complementing CHO-K1/pro⁻GAT⁻	131, 200
Chinese hamster lung cell	Glucosamine	D-glucosamine or D-galactosamine		189
Chinese hamster lung fibroblast line	asn/ts	Auxotrophic for asparagine at 39°, prototrophic at 35°C	Defective in asparagly-tRNA synthetase at 39°C	286
V-79	pur–1	Like ade⁻A	Amidophosphoribosyltransferase	59
	gln⁻	Glutamine		31
	ght	Glycine-hypoxanthine-thymidine	Folylpolyglutamate synthetase	33, 273
	urd⁻	Uridine		33
	gal⁻	Glucose; inability to utilize galactose		33, 268, 269
CCL16 (Don)	B2/Res⁻	Respiration-deficient, auxotrophic for CO_2 and asparagine, requiring high glucose supply for glycolysis	NADH-Coenzyme	46, 47, 53, 246
	B9/Res⁻	Like B2/Res⁻	Succinate dehydrogenase	261
	asn	Asparagine	Asparagine synthetase	71
	arg⁻	Arginine requirement can be satisfied by argininosuccinate but not by citrulline	Argininosuccinate synthetase	25

[a] For methods in isolating auxotrophic mutant cells, see (133, 274). No distinction has been made between mutant and variant. All the mutants listed have been characterized at least in part as to their specific nutritional requirements, enzymes affected, reverse mutation frequencies, genetic complementation, dominance and recessiveness, gene mapping, etc.

Replica Plating

The development of a replica plating procedure for mammalian cells has greatly extended the range of mutations that have become available for genetic analysis (264). One example of mutants that have been produced by this means is the UV-sensitive mutant of the CHO cell which has been demonstrated to have lost important features of its repair system for damage produced by ultraviolet radiation (266).

Gene Amplification in Drug-Resistant Mutants

An interesting phenomenon bearing on the nature of drug-resistant mammalian cells has been reported by the laboratory of Schimke (1, 247). They found that when mouse cells were repeatedly exposed to increased concentrations of the chemotherapeutic drug methotrexate (4-amino analog of folic acid), highly resistant cells were obtained that had a higher rate of synthesis of dihydrofolate reductase, the target enzyme of methotrexate. Molecular analysis revealed that several hundred copies of the gene coding for dihydrofolate reductase were present in the resistant cells. Apparently, the higher resistance resulted from gene duplication followed by stepwise selection in the presence of increased concentrations of the drug (2). A similar phenomenon of gene amplification was found in CHO-K1 cells (184). This finding is extremely important on a fundamental level as well as in contributing to an understanding of the nature of the resistance that some tumor cells develop in the course of chemotherapeutic treatment. Improved design of treatment may well result from such studies.

Regulatory Mutants and Studies in Gene Regulation

Of particular interest are gene mutations which affect regulatory mechanisms. One example of such application lies in the control of cholesterol synthesis in mammalian cells, the phenomenon underlying the disease atherosclerosis. The problem of isolating regulatory mutants was solved in the following fashion: analogs of cholesterol were examined to find one sufficiently unlike cholesterol in structure that it could not fulfill the function necessary for cell reproduction of specific incorporation in the membrane, yet similar enough to cholesterol to trigger the regulatory system to shut off cholesterol biosynthesis in the presence of an excess of this analog.

25-hydroxy-cholesterol fulfilled these requisites (257). Therefore, a mutagenized cell population was placed in cholesterol-free medium to which 25-hydroxy-cholesterol was added. Under these circumstances, no cell could form a colony unless it synthesized its own cholesterol. However, the cells with normal cholesterol regulation, perceiving the presence of an excess of 25-hydroxy-cholesterol, turned off their biosynthetic pathway and consequently failed to reproduce and form colonies. Only those mutants in

which the regulatory system had been inactivated were unable to shut off cholesterol synthesis even in the presence of the 25-hydroxy analog. Therefore, these cells alone formed colonies. Biochemical examination confirmed that such clones had, indeed, been damaged in some step of the cholesterol regulatory pathway (257, 258). This approach promises important insights into the biochemical genetics of cholesterol regulation in mammalian cells and the pathology of atherosclerosis (259). It also appears applicable to many other mammalian regulatory systems.

A number of other new approaches to the study of gene regulation in somatic mammalian cells have appeared which can only be mentioned in passing: Peterson & Weiss (209) and Kao & Puck (132) have described activation of silent genes of one cell species by incorporation of particular chromosomes from another species into a hybrid cell. Similarly, regulation of expression of genes for cell surface antigens on single human chromosomes contained in a human-CHO hybrid by appropriate Chinese hamster genes, and the coordinate activation or inactivation of these human genes, have been well-documented (112). The elegant studies of Hood (94) demonstrating that gene transpositions are necessary for maturation and production of specific antibodies by leukocytes constitute a landmark in somatic cell genetic studies. The production of single polypeptides containing multiple enzyme activities, produced by the action of contiguous genes, appears to be an important modulating mechanism of genetic activity (41). Finally, the use of chemical agents like azacytidine in controlling gene expression by changing DNA methylation (118, 234, 244), steroid hormones like estradiol and progesterone in inducing specific protein synthesis through interactions involving cytoplasmic receptors with specific chromatin sites (74, 103, 263), and cyclic AMP in regulating specific biosynthesis (68, 175, 294) have opened up new areas in the understanding of gene expression. There seems little doubt that major understanding of the differentiation processes in normal and disease situations will be forthcoming as a result of these advances.

SOMATIC CELL FUSION AND HYBRID STUDIES

General Considerations

The discovery of the phenomenon of somatic cell fusion provided a quantum leap in the powers of somatic cell genetics (for review see 55, 90, 238). The development of cell fusion as a standard technique in somatic cell genetics is the result of successive contributions by the laboratories of Okada (187), Barski (6), Ephrussi (262) and Harris (91), each of whom made significant contributions to this operation, which has become so powerful an adjunct in genetic analysis. The early fusions were mediated by

irradiated Sendai virus. Later, substitution of chemical agents like polyethylene glycol as inducers of fusion further increased the ease of this procedure (211). Indeed, cell fusion gave somatic cell genetics new powers denied to classical genetic studies in which reproductive mating is possible only between members of the same species. In contrast, fusion of somatic cells can be effected between cells of different species, genera, families and perhaps even more remotely related organisms. Thus, it becomes possible to introduce into particular cells genetic material from widely diverse living forms, so that much greater possibilities of genomes can be prepared. The variety of experiments now available in somatic cell genetics forms a cornucopia of opportunity for study of gene and chromosome behavior.

Dominance and Complementation Analysis

The cell fusion technique made it a simple matter to determine whether dominance or recessive relationships exist between two allelic genes by testing the phenotype of the appropriate fused cell (122, 124). Equally important was the ability to carry out complementation analysis, which is routinely performed as follows: A cell population is mutagenized and then subjected to a selection procedure designed to yield mutants of a particular phenotype. The resulting mutant clones are picked; new stocks are developed and tested to ensure that they display the mutant phenotype in stable fashion. The question of how many different genotypes are represented by this collection of clones with the same phenotype is answered simply by progressive pairwise fusing of the recessive mutant clones with each other and testing the resultant hybrids for the presence of the genetic characteristic under study. The absence of complementation implies that two mutants are defective in the same genes, both of which are required for the given phenotype to be expressed. In this way, the total number of complementation groups can be established, and indicates the number of genes, any one of which can be mutated to prevent the appearance of the given phenotype. Thus, the number of independent, gene-controlled biochemical steps in the given pathway can be approximated.

The first application of this complementation analysis to mammalian cells was demonstrated with the glycine-requiring gly^- mutants in CHO-K1 cells (122). Similar complementation analysis conducted in large numbers of purine-requiring mutants of CHO-K1 cells resolved at least nine complementation classes (206).

The sequence of biochemical steps in the given metabolic pathway can then be determined by means of feeding experiments, in which different metabolites are added to the nutrient medium for each of the different complementation groups to determine which nutrients will overcome the block, and by means of biochemical analysis of enzymatic steps in each

complementation group as compared to the wild-type cell. Such biochemical studies have been carried out for the pathways involved in biosynthesis of glycine (122), purines (100a, 185, 200–202, 205, 206, 282), and pyrimidines (40, 41, 203, 204, 265). Thus, there is now available a reasonably general method for investigating the genetic-biochemical pathway for a wide variety of systems for which one can prepare and select mutants of a desired phenotype.

Gene Expression in Cell Hybrids

By combining different genomes into the same cell, the regulation and expression of specific genes in the resulting hybrid cell can be studied. For those genes coding for specific functions essential for cell survival (housekeeping genes), codominant or coexpression gene activity is generally observed (153). For the genes coding for functions unique to differentiated states in particular tissue cells (differentiation genes), the following three types of gene activity have been found in the cell hybrids: (*a*) loss of differentiation gene activity, like the pigment production in the melanoma/fibroblast hybrids; (*b*) maintenance of differentiation gene activity, like a number of neuronal-specific functions and enzymes; and (*c*) induction of new differentiation gene activity not previously expressed, like the production of mouse albumin in hybrids formed between mouse fibroblasts and rat hepatoma cells (for review see 42, 55, 238). In addition, Fougere & Weiss (62) reported another type of differentiation gene activity in hybrids of melanoma and hepatoma cells, called "phenotypic exclusion," in which only one of the two characteristics, pigment or albumin, is expressed, and the character that is expressed alternates in successive subclonings.

Recent advances in the use of two-dimensional electrophoresis-electrofocusing gels have proved particularly useful in study of gene expression in hybrids (15, 156, 166 , 251).

Single Human Chromosome Hybrids

When rodent cells are fused with diploid human cells, the hybrids usually lose human chromosomes, so that often only one or very few human chromosomes remain in the hybrids (130, 288). These retained human chromosomes can be identified by both cytogenetic and isozyme analyses (125). Hybrids with single human chromosomes have been established for chromosomes 11 (125), 12 (125, 141), 21 (179), 14 (38), and X (174).

The use of auxotrophic cell mutants to cause retention of specific human chromosomes complementing the nutritional deficiencies of the mutants has permitted preparation of a variety of hybrids and assignment of genes to particular human chromosomes. Thus, the gene for serine-hydroxy-

methyl transferase has been assigned to human chromosome 12 (116, 125, 141) and genes in the adenine biosynthetic pathway to human chromosome 21 (179). The method appears to be fairly general and auxotrophic mutants have been used to prepare other hybrids with single or particular combinations of human chromosomes (113).

Human chromosome loss is more rapid and extensive in human-CHO hybrids than in human-mouse hybrids (130). The mechanism determining the direction and extent of chromosome loss is not yet elucidated, although it has been proposed that the large differences in generation time between CHO and human cells may contribute to the extensive loss of human chromosomes in these hybrids (130). Other studies have shown that the determining factor may in some cases involve the cell type rather than the species from which the cells are derived. Thus, when normal mouse cells are fused with some human permanent cell lines, mouse chromosomes are lost from the hybrids (37).

Another development which aids in establishment of hybrids with single or small numbers of human chromosomes involves fusion of whole rodent cells with human microcells which contain a few human chromosomes. Microcells can be prepared either by arrest of human cells like HeLa with nitrous oxide in a high pressure chamber followed by cold treatment and 37° incubation (105), or by colcemid treatment of normal human fibroblasts to produce micronuclei followed by enucleation with centrifugation in the presence of cytochalasin B (54, 63, 64). Both methods have been successfully used for preparation of mouse cell hybrids containing single or small numbers of human chromosomes (170, 281).

Occasionally, stable single chromosome hybrids have been prepared without the use of selective markers (125). These hybrids have been useful in a variety of different genetic approaches as described in subsequent sections.

Premature Chromosome Condensation and Chemotherapy

An interesting case of hybridization was developed by Johnson and Rao (106) in which CHO cells phased in different parts of the life cycle were fused with each other. These studies demonstrated that cells in different parts of the life cycle could exert dominance relationships over cells in other phases. Thus, the nucleus from S phase cells could induce premature DNA synthesis in nuclei of cells from G_1 phase (233). Most striking was the fact that a mitotic cell fused with a cell in any other part of the life cycle causes the latter to undergo premature chromosome condensation (PCC) (106). In this way, heterokaryons prepared from a mitotic cell and a G_1 cell exhibit condensation of the latter's chromosomes so that they become visible under the light microscope. Since the chromosomes of the G_1 cell have not yet

doubled, they form condensed single rather than double stranded chromosomes. Mitotic cell factors that induce chromosome condensation in interphase cells have been shown to become associated with the chromatin of the latter cell, and are sensitive both to DNAase and protease (44). This PCC phenomenon has found a number of interesting applications. One clinical study has demonstrated its usefulness as an early indicator of relapse in human acute leukemia before other clinical signs are apparent (92). Thus, the physician is furnished with a more accurate guide for ascertaining the imminence of a relapse and can readminister chemotherapy in more effective fashion.

CELL SURFACE ANTIGENS

Somatic mammalian cells contain a wide variety of macromolecular membrane structures. Some of these, like the HLA antigens, appear in all or most tissue cells, and are responsible for transplantation rejection phenomena (135), a subject which will not be treated in this paper. Other cell surface macromolecules, however, have a high degree of tissue specificity and presumably are connected with the specific exchange of information between cells of particular differentiation states and their molecular environment.

Extensive literature has accumulated dealing with such cell surface receptors. We shall confine our treatment to a particular somatic cell genetic approach which is effective for a variety of receptors and other cell surface antigenic macromolecules. This methodology has the advantage that particular cell surface macromolecules and the loci of their genetic determinants are identified simultaneously. In this way, a powerful approach is afforded for study of the genetics, biochemistry, and immunology of such molecules. The approach is embodied in the following steps:

1. It was demonstrated that cell surface antigens of the human and Chinese hamster somatic cells display great specificity with respect to antibody binding and cell killing by antibody and complement. Thus, in the presence of complement extremely small concentrations of antisera formed against human cells in the rabbit will kill more than 99% of the human cells used as antigen, whereas even much higher concentrations of this antiserum fail to produce measurable killing of Chinese hamster cells under similar conditions (186).

2. A particular human tissue cell, for example normal human fibroblasts, can be injected into rabbits to produce an antiserum which, in the presence of complement, is highly lethal to the human fibroblasts, but relatively inert to CHO cells. This antiserum is then tested against various human-Chinese hamster hybrids containing specific human chromosomes. In the present

example it was found that the hybrid containing the single human chromosome 12 is unaffected by this antiserum even in fairly high concentrations. It may then be concluded that the human fibroblast contains no antigens in common with those that human chromosome 12 elicits on the cell surface of the hybrid containing this human chromosome (117).

3. However, when the same experiment is carried out on the hybrid containing the single human chromosome 11, extensive cell killing is achieved by very low concentrations of antiserum (125). It may be concluded that the normal human fibroblast membrane does share antigens in common with those that human chromosome 11 elicits in hybrids containing it as the only human chromosome. Other investigation also found the existence of cytotoxic antigens associated with chromosome 11 (18, 19).

4. This antiserum now can be adsorbed exhaustively with hybrid cells containing human chromosome 11, and then tested for its killing action on hybrids containing other human chromosomes using the same general procedure.

5. Having identified chromosome 11 as one containing loci responsible for production of antigenic activity similar to components of the human fibroblast membrane, it becomes necessary to resolve this activity into individual antigens. Such resolution can be achieved by several methods which have been described. At least five different cell surface antigens have been characterized as requiring loci on human chromosome 11 for their expression. Antisera specific to each antigen can then be prepared (111, 115).

6. By means of immunofluorescence or horseradish peroxidase staining such antisera can be applied directly to tissue sections. Thus, it has been shown that loci on human chromosome 12 produce antigens present on the human brain, while antigens associated with chromosome 17 appear on human kidney cells (Puck, Nielson, and Kao, unpublished data).

7. Mutants lacking particular cell surface antigens can readily be obtained by treating mutagenized cell populations with a specific antiserum and complement. Only cells which have lost the antigen in question form colonies which can then be picked and established as mutant stocks (117).

8. These antigens can be studied further by genetic complementation of appropriate mutants, as already described, and by biochemical analysis. It is interesting that some of these antigens require not only loci on human chromosome 11, but also particular loci of the Chinese hamster genome for their expression (111, 112).

9. The deficient mutants so produced can then be studied cytogenetically, particularly where the mutagens employed are ones with high efficiency of deletion production. The loci involved can be mapped as described

in the following section. The effect on the expression of these antigens by hormones and agents like cyclic AMP can also be studied.

10. Finally, other tissues, like cells from the human kidney and brain, can be injected into rabbits and antisera obtained and used just as has been described for antisera to the human fibroblast. Indeed, hybrids themselves containing particular human chromosomes can also be injected into rabbits and antisera prepared in this fashion, although often such antisera have weaker titers than those prepared by the alternative method. In this way, large numbers of cell surface antigens involving many different tissues can be subjected to genetic analysis.

The approach described in this section has also been used to study the receptor for transferrin which has been demonstrated to lie on human chromosome 3 (177).

This discussion has presupposed that the actions of genes on the different human chromosomes will conform to a simple additive model. Obviously, this may not always be the case. The approach described should reveal the existence of complex chromosomal interactions by the fact that specific gene expression from loci on the single chromosome may be altered when that chromosome is present in a hybrid together with other specific human chromosomes. However, the existence of such regulatory phenomena would automatically be revealed by the approach described here. These experiments then promise to be useful in elucidation of genetic control of cell surface structures in differentiation.

CHROMOSOMAL AND GENE STRUCTURE

General Considerations

Application of the new techniques of molecular biology and recombinant DNA have revealed totally unexpected aspects of mammalian cell genetic structure. The relative simplicity of the genetic apparatus of *E. coli* had led to the expectation that mammalian cells would be different only in degree from the microorganisms. On the contrary, the bewildering variety of relationships uncovered makes it clear that differentiation and development in somatic mammalian cells display changes in genome of such magnitude that a whole new era in genetic analysis must now begin.

We mention only in passing the following: the demonstration of the complexity of the structure of the eukaryotic nuclei; the existence of many different kinds of repetitive DNA sequences interspersed with unique sequences (39, 102, 249); the existence of gene families (61, 176); the still unelucidated mechanism of chromosome condensation (67); the existence of specific chromosome inactivation and its association with an entity like the Barr body (5); the demonstration of the existence of introns (101, 148)

and special DNA sequences like the TATA box and others which flank the coding regions (16); the phenomena of oncogenes (9) and pseudogenes (176); the actions of transposable elements (60, 213); and the demonstration that differentiation of immunoglobin requires specific rearrangements of DNA sequences to produce the large variety of antibodies known to be coded for in the mammalian genome. (94).

In this section we restrict our discussion to only a few of the newer methods now available for exploring mammalian genome structure, with the aid of techniques like sequence determination and the recombinant DNA methodologies.

Recombinant DNA and Somatic Cell Genetics

Recent developments in recombinant DNA technology have opened up new dimensions for genetic analysis. These technologies have been combined with somatic cell genetic analysis to increase resolving power from millions to thousands of base pairs (bp). With the added availability of rapid DNA sequencing methods (160, 245), analysis can be further refined to the level of individual base pairs for studies of somatic cell genetics in general and the human genome in particular.

Construction of the Total Human Genomic Library

In 1978, a human genomic DNA library was first constructed from human fetal liver DNA using the bacteriophage λ Charon 4A as the vector (145). Human DNA fragments of 15 to 20 kb were fractionated and ligated into the phage arms for in vitro packaging. It has been estimated that 8×10^5 recombinant phage clones are required for a complete "library" having 99% or greater probability of including every sequence present in the human genome (Maniatis et al 1978). Thus, 10^6 in vitro packaged phage clones were prepared and each amplified 10^6 fold to establish a permanent human genomic DNA library. This library has been used to isolate the first human genomic β-globin gene sequences (66, 158). Human genomic libraries containing smaller DNA fragments can also be constructed using vectors such as plasmid pBR322 (11).

Similarly, cDNA derived from polysome-bound poly(A)$^+$ RNA isolated from human cells can be used to construct libraries using a bacterial plasmid as vector (293). Since a eukaryotic cell contains 10,000–30,000 different mRNA sequences, a complete cDNA library needs a much smaller number of clones than does a complete genomic library. A cDNA library is extremely useful in studying gene expression in different tissues (36) and developmental stages (190).

Construction of a DNA Library from Individual Human Chromosomes

Two general approaches have been developed to construct a DNA library for individual human chromosomes: (*a*) use of the fluorescent-activated cell sorter to fractionate individual human metaphase chromosomes, or (*b*) use of somatic cell hybrids containing a single human chromosome.

The first approach has been used to construct DNA libraries for the human X chromosome (43). Recent progress in flow cytometry using a fluorescent-activitated cell sorter (FACS-II) can resolve the human chromosome into as many as 20 peaks (298). While most of these peaks contain a single specific human chromosome, some contain two or more chromosomes. For example, the peak containing human chromosomes 9–10–11–12 is difficult to resolve. Moreover, chromosomes 1–2, 13–14–15, 17–18, and 21–22 cannot always be separated completely in some samples. Finally, the fractionated chromosomes are not absolutely pure. Thus, the fraction taken from the peak containing the X-chromosome also contained about 10% of chromosomes 7 and 8 (43). Despite these limitations, this approach offers great promise for eventual construction of complete DNA libraries for most, if not all, of the human chromosomes.

Using the fractionated X-chromosome, Davies et al (43) constructed a library of 50,000 recombinant phage λgtWESλB. Among 30 phage analyzed, 6 contained single copy sequences derived from the X chromosome; the rest contained repetitive sequences and one apparently was not of X chromosomal origin. Because of the possibility of chromosomal impurity it is necessary to test such preparations to ensure their chromosomal origin.

The second approach has been applied to human chromosome 11 (85) and has been extended to chromosomes 12 and 21 (144). Human-CHO cell hybrids containing the single human chromosome 11 were used to provide DNA fragments from that chromosome. DNA fragments of 15 to 20 kb in length were ligated to λ Charon 4A phage arms. The corresponding clones of recombinants obtained after in vitro packaging contain both CHO and human chromosome 11 DNA, which need to be distinguished.

A method for accomplishing this was developed using species-specific, middle-repetitive DNA sequences (165). Only phages containing human DNA will hybridize to ^{32}P-labelled, repetitive human DNA probes, while phages containing CHO DNA fail to hybridize to such a probe. Using this method, phage containing human DNA fragments from human chromosome 11 were isolated.

Unique sequences within the human insert can be identified and isolated by additional restriction enzyme digestion of these original isolates to produce subfragments, since the subfragments that fail to hybridize to human

repetitive DNA are likely to contain unique sequences. Regional assignment of these unique subfragments can then be made by hybridizing them to the DNA from a series of cell hybrids containing various terminal deletions of human chromosome 11 (108). Thus, human DNA fragments inserted in particular phage clones were assigned to the following regions of chromosome 11: pter-p13 (1 phage); p13-p1208 (2 phage); and p11-q13 (2 phage) (85). (See section on mapping human genes by somatic cell hybrids and recombinant DNA probes).

This approach appears to be general and can be applied to other human chromosomes for which cell hybrids containing single human chromosomes have been constructed, including chromosomes 12 (125, 141), 21 (179), 14 (38), and X (73, 248, 296). This approach should be especially useful for those chromosomes that are difficult to separate by flow cytometry, such as chromosomes 9, 10, 11, and 12 (298).

Another approach, competitive liquid hybridization, has been used to isolate chromosome-specific DNA for the two sex chromosomes. Exhaustive reannealing between DNAs prepared from male and female cells allowed isolation of Y chromosome-specific, repetitive DNA (139). However, this procedure permits isolation of only a fraction (7–11%) of the total DNA sequences from the Y chromosome. Furthermore, it cannot isolate unique DNA sequences that are not adjacent to repetitive sequences.

Hybridization in solution has also been used to isolate DNA from the human X chromosome (188, 248). In these studies, human-mouse cell hybrids containing a single intact (248) or partial (188) human X chromosome were used. Competitive reassociation was carried out between the DNA from hybrid cells and the mouse DNA. The unbound DNA was considered to be derived from the human X chromosome. The limitation of this approach is that the human X chromosome DNA has nearly 50% homology with the mouse genomic DNA. Since the isolated human DNA represents only a part of the total DNA from the X chromosome, a library constructed from such DNA contains only a fraction of the human X chromosome sequences.

GENE TRANSFER

The exchange of genetic material through cell mating first became possible in the cultured somatic cells by the discovery of cell fusion. More recently, transfer of specific genes from one cell to another by purified DNA or by use of metaphase chromosomes has been achieved. Although the frequency of transfer by these methods was initially lower than that achieved by cell fusion for most cell types, this frequency has been increased (151), and by

use of microinjection techniques (24) has achieved impressively high efficiency.

Gene transfer in mammalian cells using purified DNA was first attempted by Szybalska and Szybalski (270, 271) using the 8-azaguanine resistance marker and the HAT selection system. These early experiments yielded inconclusive results. The breakthrough in DNA-mediated gene transfer in mammalian cells came with the adoption of calcium phosphate-precipitated DNA procedures (4, 78, 79) and the use of the mouse Ltk$^-$ cell as recipient (155, 207, 291). The source of DNA used in these originated from herpes simplex virus (4, 291) or from total cellular DNA from tissues and cultured cells (292). Although some successful experiments have been reported using other recipient cells, the transfer frequency in general is much lower than that achieved with the mouse Ltk$^-$ cells (152, 208).

In DNA-mediated transfer experiments, cotransfer of unselected markers has also been observed and the frequency is surprisingly high (290). In most cases, a high proportion of transferrents carrying unselected markers occurs when DNA from selectable and unselectable markers is mixed and coprecipitated with calcium phosphate, before addition to the recipient cell culture. This procedure provides an effective means for transfer of markers without selection, and their subsequent integration into the recipient genome.

Gene transfer mediated by metaphase chromosomes was first demonstrated by McBride and Ozer (161). This process was confirmed and extended to a large variety of mammalian cells as recipients (162). The frequency of transfer ranged between 10^{-7} and 10^{-5} per cell. The frequency can be increased by treating recipient cells with DMSO, increasing the time of interaction between chromosomes and the cell, or increasing the chromosome concentration (151). Under optimal conditions and using mouse Ltk$^-$ clone D cells as the recipient, transfer frequency of thymidine kinase (TK) or dihydrofolate reductase genes reaches approximately 10^{-4} per recipient cell (151). Some transferrents derived from either DNA or chromosome-mediated gene transfer lose their transferred genetic material at a rapid rate whereas others are stable, retaining their acquired genetic material for many generations (136, 291).

High frequency of gene transfer has recently been achieved by microinjection of purified genes into mammalian cells (24). The purified TK gene has been microinjected into mouse Ltk$^-$ cells at a rate of 1000 cells per hour. About 20% of the injected cells were found to be transferrents. It is important to note that transferrents can be found only if the genes are injected into the nucleus of the recipient cell. It thus appears that either the nuclear membrane acts as a barrier preventing penetrance of the input DNA, or the input DNA is at greater risk of being degraded in the cytoplasm. This

finding may explain the low frequency of gene transfer that occurs when DNA or metaphase chromosomes are added directly to a cell culture.

So far, no successful transfer has been reported using normal diploid human cells as recipient by DNA or metaphase chromosomes. It appears that microinjection may be the most promising route for introducing genes into human cells with high frequency.

Cloned genes were microinjected into the pronucleus of mouse eggs (283) and subsequently expressed themselves by production of functional proteins in late fetal life. These advances promise highly illuminating in vivo studies of gene regulation during developmental processes.

HUMAN GENE MAPPING AND USE OF HUMAN DNA FRAGMENTS AS GENETIC MARKERS

General Considerations

There are two principal reasons why it becomes necessary to be able to map the human genes with the highest possible resolving power. The first reason is an intensely practical one: at the present time, there are approximately 100 different genetic diseases which can be reliably diagnosed in the fetus early in pregnancy (168). Prospective parents can now be offered the possibility of terminating those pregnancies that involve severe defects, the only medical intervention possible today. However, on the horizon there appear two other distinct possibilities. The steadily increasing powers of gene transfer make the prospect of introducing normal genes to compensate for defective ones an increasingly practical possibility. A second approach involves the possibility that as we come to understand the metabolic basis for genetic diseases, biochemical manipulation of metabolic states to overcome pathological consequences of the defective genes may become feasible. In either case, it is necessary to be able to diagnose genetic defects as early as possible in pregnancy.

While the ability to diagnose approximately 100 different genetic diseases *in utero* seems a major accomplishment compared to the situation a relatively short time ago when virtually none of these diagnoses was possible, the actual number of well-characterized human genetic diseases numbers in the thousands. Thus, the bulk of these diseases lies outside of our present powers of prenatal diagnosis. However, by means of recombinant DNA techniques, it becomes possible to develop new methods of diagnosis of genetic defects which give promise of vastly expanding the number of such prenatal identifications. Accurate mapping of the human genes is an important step in such techniques.

Important as it may be to diagnose genetic diseases more effectively, the second reason why high resolution gene mapping is necessary is even more

weighty. Everything we know suggests that control mechanisms which regulate gene expression are intimately related to the constitution of the specific DNA sequences lying between or within the gene coding regions. If we can ascertain for any gene its chromosomal position and its specific regulatory nucleotide sequences, it should be possible to deduce the strategy governing its expression in normal human development and the defects which arise in specific diseases.

The methodologies of recombinant DNA offer means of production of specific DNA probes for detection of suspected genetic defects (120, 191, 192). Sometimes preparation of such probes by the conventional methods, which involve isolation of the appropriate messenger RNA, is difficult or impossible.

It has been demonstrated that DNA probes can be prepared from specific human chromosomes and mapped regionally on these chromosomes provided that the corresponding single human chromosome hybrid, from which appropriate terminal deletion mutants can be prepared, is available (85). If this process can be extended to fine structure mapping it should be possible to map such probes with great resolving power without the need for isolation of the appropriate messenger.

Mapping of Human Genes by Somatic Cell Hybrids

On the basis of the chiasma frequency and gene density in the mouse, it has been calculated that the human genome contains approximately 60,000 genes (169) distributed among the 22 autosomes and the X and Y chromosomes. The first human gene to be mapped was the color blindness trait assigned to the X chromosome by Wilson in 1911. During 1911–1967, human gene assignments were carried out mainly by pedigree analysis. About 100 genes were assigned to the X chromosome and only a few autosomal genes were assigned to a specific chromosome (168).

Important breakthroughs in human gene mapping evolved, including development of somatic cell genetic cell fusion and the demonstration of segregation of human chromosomes in the human-rodent cell hybrids (288); chromosome banding techniques which permit recognition of specific regions of human chromosomes; and the cytogenetic and isozyme identification of human chromosomes in hybrids. At present, every human chromosome except the Y has at least one isozyme marker and most chromosomes have separate isozyme markers on both arms.

These technical advances have permitted tremendous progress in the past 10 years in mapping human genes. More than 400 genes have been mapped, of which a great majority are autosomal genes, and mapping continues at a rate of about 40 to 50 new genes per year (167, 169, 241).

For rapid chromosomal assignment of human genes, a clone panel can be used comprised of a collection of human-rodent cell hybrids containing specific combinations of human chromosomes. Thus, genes coding for unique human markers can be mapped to one of the human chromosomes matching its pattern of presence or absence with that of individual human chromosomes in the panel.

In order to increase such gene assignments, efforts have been made to (*a*) characterize more enzymes physically, chemically, and immunologically, to distinguish human and rodent gene products; (*b*) devise methods for inducing human gene activities which are not normally expressed in hybrids; (*c*) construct new clone panels in both human-mouse and human-Chinese hamster hybrids for the comparison and confirmation of enzyme expression; and (*d*) construct a complete set of cell hybrids each containing a single, unique human chromosome which confirms the gene assignments made by clone panels.

After a gene has been assigned to a specific chromosome, its location within the chromosome must be determined. Regional mapping has been carried out mainly by cytogenetic analysis of cell hybrids containing translocations or deletions of specific human chromosomes. In some cases, gene dosage differences can be detected by the change from the normal amount of the gene product in cells from patients with identifiable monosomies or trisomies (235, 236). As a source of human cells with specific chromosomal aberrations the collection in the Human Genetic Mutant Cell Repository, in Camden, New Jersey, is extremely valuable (99).

For extensive and systematic regional mapping of the human genome, a large variety of chromosomal breaks and deletions widely distributed in the karyotype is required. For this purpose, chromosome-breaking agents have been used to induce chromosome deletions for regional mapping of chromosomes 1 and X (20, 21, 75–77) and for chromosome 11 (126) and 12 (141). Human chromosome breakage can be induced either by treatment with human cells before they are fused with other cells, or by treating the hybrids containing human chromosomes. The former procedure has been shown to produce a greater variety and frequency of chromosome breakage (141).

Regional mapping can also be carried out by statistical analysis of the segregating data as described by Goss & Harris with human chromosomes 1 and X (75–77), and by Law & Kao (141–143) with chromosome 12. In these analyses, the gene order and the relative distances can be calculated using the radiation-induced segregation frequency of syntenic markers in the hybrids and application of target theory, which states that the size of the target (i.e. the distance between two loci) is directly proportional to the segregation frequency of the two genes caused by radiation-induced

chromosomal breakage. In general, the gene order established by this method agrees with that obtained by cytogenetic mapping (76, 77, 142, 143).

The basic difference between the cytogenetic and statistical mapping analyses is that the former method assigns mapping positions to the highly condensed metaphase chromosomes, whereas the latter measures relative distances between genes in the extended interphase chromosomes. Thus, the gene map based on the statistical mapping analysis should represent more closely the classical gene map based on the recombinant frequencies resulting from crossing over during meiosis.

For rapid regional mapping of a particular chromosome, a set of deletion hybrids can be constructed, each containing a different terminal deletion of that chromosome. For example, hybrid clone A_L-J1, which contains the single human chromosome 11, was treated with chromosome-breaking agents (126), and subclones isolated and characterized. A set of five deletion hybrids with various deletions on chromosome 11 was established and used for rapid regional mapping of genes on this chromsome (84, 108, 172).

In addition, deletion hybrids identified by various combinations of marker losses can also be used for regional mapping. These deletion hybrids can be characterized by the presence or absence of isozymes or other markers on the chromosome without the need for detailed cytogenetic analysis. Such marker-deletion hybrids have been established for chromosomes X (8) and 12 (141–143). The advantage of marker-deletion hybrids over the cytogenetic-deletion hybrids is that the former can extend the resolving power to a region beyond that currently possible by cytogenetic techniques.

Use of Recombinant DNA Probes in Gene Mapping

Recombinant DNA technology has provided probes consisting of labelled DNA segments containing specific human genes for use in molecular hybridization experiments with genomic DNA from test cells to be examined. Such cells include somatic cell hybrids containing specific combinations of human chromosomes. This allows rapid detection of the presence of specific genes without the need for their expression. Further developments, including restriction endonuclease digestion and Southern blot techniques, have made the analysis even more definitive and accurate.

Mapping of structural gene sequences was first carried out with the human α- and β-globin genes. Using a series of human-mouse cell hybrids, the α-globin gene was assigned to human chromosome 16 (49) and the β-globin gene complex to chromosome 11 (48). In these experiments, nucleic acid hybridization in solution was used.

Filter hybridization of restricted DNA fragments with highly radioactive probes was first applied to the assignment of the β-globin gene complex to human chromosome 11 (84, 146, 252). cDNA probes of the β-globin gene complex were used, and all three studies confirmed the assignment of the gene to chromosome 11.

A variety of approaches was used for achieving regional mapping. Scott et al (252) used a human-mouse hybrid with a single translocated human chromosome that contained a large part of chromosome 11 (pter - q23) and a small part of the X chromosome (q25–26 - qter). Since the probe hybridized molecularly to DNA from this hybrid, but not with DNA from hybrid clones that had lost this translocated human chromosome, it was concluded that the distal region of the long arm (11q23 - qter) was not the site for the β-globin complex. Using DNA extracted from fractionated human metaphase chromosome, Lebo and coworkers (146) assigned the β-globin gene complex to 11p12-pter. The most refined regional mapping of the β-globin gene complex was accomplished by Gusella (84), using a series of human-CHO cell hybrids containing various deletions of chromosome 11 (108, 126). The β-globin gene was assigned to the region 11p1205–11p1208, a segment of about 4500 kb, 135 times larger than the β-globin gene complex (Fritsch et al 1980).

Other gene assignments by means of filter hybridization with radioactive probes include the human growth hormone gene complex to chromosome 17 (195), the insulin gene to chromosome 11 (193), proopiocortin gene to chromosome 2 (196), prolactin gene to chromosome 6 (194), and interferon gene complex to chromosome 9 (197). More gene assignments will be made by these techniques as appropriate probes become available.

It is important to point out that probes developed from genes of other animal species can be used for mapping human gene sequences. The high degree of sequence homology of many functional genes among different species permits effective cross hybridization between the DNA of human and other species. With the use of appropriate restriction enzymes, fragments that differ in size can be prepared from the two species and used as species-specific probes (193–197).

In situ molecular hybridization constitutes another promising development in gene mapping. When the DNA probes are labeled with ^3H at high specific activity by nick translation and hybridized to mitotic chromosome preparations in the presence of dextran sulfate, attachment of the probe to homologous regions on the chromosomes occurs (89, 157). This approach promises to be extremely useful for both chromosomal and regional mapping; even unique sequences can be identified by this means.

Human DNA probes as Genetic Markers

For extensive mapping of the human genome, a general procedure has been proposed using human DNA fragments as genetic markers to define particular human genetic loci. Polymorphic derivatives in DNA sequences in particular individuals can then be readily detected (14). Such polymorphisms can result from DNA alterations, like deletions, additions, or substitutions of single base pairs, or blocks of aberrant DNA occurring either inside or outside the recognition sites. In order to be useful for linkage analysis, these DNA fragments should include specific restriction enzyme recognition sites so that after DNA digestion, restriction fragments of different sizes are obtained from the polymorphic individuals. It has been estimated that 150–200 well-spaced DNA markers will be needed to cover the entire human genome. The method does not require understanding the biochemical nature of the disease for mapping its genetic determinant.

The DNA fragments serving as genetic markers can be isolated from the human genomic DNA library (145). Since the great majority of such phages contain interspersed, repetitive human sequences, it is necessary to screen for those containing only unique sequences for detecting polymorphism. Alternatively, the human insert in the phage can be cleaved with various restriction enzymes, and subfragments that contain unique sequences within the insert can be identified.

About 1% of the recombinant phages in the human genomic library contain unique human sequences (123). Chromosomal localizations of two such human inserts were made by blot hybridization and synteny analysis using a series of human-CHO-K1 cell hybrids containing unique combinations of human chromosomes. The human DNA fragment of 14.7 kb contained in one recombinant phage was assigned to human chromosome 22, and another fragment of 11.4 kb to chromosome 10. This isolation and mapping procedure for human unique DNA fragments appears to be simple and can be used to assign large numbers of such fragments to any human chromosome. These fragments will be highly useful as genetic markers not only for specific human chromosomes, but also for detecting polymorphism in human populations for linkage studies between various human traits and inherited diseases (14, 297). For example, a highly polymorphic human single-copy DNA fragment of 16 kb has been characterized that exhibits at least 8 variants (297). Less than 25% of the individuals examined were homozygous for this locus. The polymorphism appears to be due to rearrangements of DNA segments of various lengths. This DNA fragment has been assigned to human chromosome 14 (51).

CANCER, ONCOGENES, AND THE CYTOSKELETON

The genetic basis for cell transformation, a step preceding malignancy in mammalian cells, constitutes one of the enigmas of mammalian cell biology. While by this time there is little doubt that genetic change is an intrinsic characteristic in most cancers, the wide variety of different chromosomal alterations associated with most malignancies has presented a confusing situation.

A number of new clues now promise possible fundamental understanding. Cytogenetic analysis has revealed a variety of different malignancies to be associated with defects in one or more specific chromosomes (69). The association of chronic myelogenous leukemia with a translocation involving chromosomes 22 and 9 has been recognized for some time (183, 239). The association of the Wilms' tumor-aniridia syndrome with a deletion in the p13 region of the short arm of chromosome 11 represents a similar situation (237). The demonstration of an association between small cell cancer of the lung and a deletion in the p14-p23 region of the short arm of chromosome 3 has recently been established (289), and retinoblastoma has been shown often to be accompanied by a deletion in the q14 region of chromosome 13 (295).

These various findings suggest that specific sites on particular chromosomes possess genes needed for normal differentiation in particular tissues. A deletion in such a critical area then may prevent normal development and produce a malignancy in a particular organ. If this interpretation of the facts is correct, studies of this kind may well pinpoint chromosomal regions which determine important developmental processes in particular tissues.

One of the most puzzling phenomena of cancer involves the complexity of the transformation process. When fibroblastic cells become malignant, they undergo changes in a variety of different characteristics: the cell morphology changes from an elongated spindle shape to a compact, pleomorphic body; the membrane of these cells is often studded with protruding knob-like structures which display intense oscillatory activity so that a tremendously hyperactive membrane results (80, 95, 170a, 220, 221, 231); the characteristic fibronectin deposit around the membrane disappears (182); characteristic changes in specific active transport and particular cell surface macromolecular structures occur (221); the cells change their monolayered growth pattern to one of three-dimensional growth when attached to surfaces, and single cells acquire the ability to multiply in suspension (221); and the chromosomal constitution becomes unstable so that the frequency of abnormal chromosomal numbers increases markedly. The reason why these phenomena occur in coordinate fashion when a cell

undergoes transformation has constituted one of the basic enigmas in the field of cancer.

An important illumination of this process arose as a result of the demonstration that all of these different manifestations of transformation could be reversed by the addition of cAMP derivatives to the affected cells (95, 96, 104, 231). This process has been named reverse transformation (231). It was also demonstrated that agents like colchicine and vinblastine, under conditions where their action specifically affects cytoskeletal structures, will prevent these actions of cAMP and, indeed, will induce the morphological characteristics of transformation in normal fibroblasts (96, 231). Transmission electron microscopy demonstrated the disorganized cytoskeletal constitution in the transformed cell which is repaired after reverse transformation (212). Consequently, it was proposed that the group of transformation phenomena listed above is induced by the failure of cell functions which depend on integrity of the cytoskeletal organization (220, 221). Evidence establishing the role of the cytoskeleton in one transformation characteristic, the regulation of cell growth, has by now been put forth in a number of different laboratories (17, 95, 242, 243, 254).

The most clearly demonstrated function of cAMP in mammalian cells is the initiation of new protein phosphorylation reactions in serine and threonine residues (138). Oncogenes like the *Src* gene, on the contrary, cause cell transformation by the production of protein phosphorylation at tyrosine residues (100). It was shown that cAMP can produce reverse transformation of cells in which malignancy was previously induced by the *Src* gene of Rous sarcoma virus (225). It would seem a reasonable hypothesis, then, that normal as opposed to malignant cell behavior can be determined by the ratio of normal to the abnormal kinds of phosphorylation produced on certain key proteins within the cell. It is further tenable as a working hypothesis, though by no means established, that some of these key proteins may be intimately associated with cytoskeletal structure and function (22, 253).

Genetic analysis of these phenomena would seem to be a promising direction to take. The production of mutants of the CHO cell which resist the reverse transformation action of cAMP has been described (97, 147, 260).

It has been demonstrated (10) that under the conditions of reverse transformation, phosphorylation of a 55,000 dalton peptide is increased and a 22,000 dalton peptide is decreased in the CHO cell. A cAMP-induced phosphorylation of a 52,000 dalton peptide has also been observed by other investigators in the CHO cell, but it was concluded from analysis of a series of mutants that phosphorylation of this protein was not sufficient to account for the reverse transformation effects of cAMP derivatives on this cell (147).

In another series of cAMP-nonresponsive mutants of the CHO cell, some clones failed to show any of the typical cAMP-induced phosphorylations characteristic of the wild-type cell. However, one unresponsive mutant had lost only one (or possibly two) of its phosphorylation responses to cAMP in the course of loss of growth control. These data have been provisionally interpreted to indicate that mutants which have lost the growth control response to cAMP and all of its phosphorylation responses are defective in one of the early steps in the biochemical pathways initiated by cAMP. However, mutants which have lost the growth control response, and have lost only one (or two) phosphorylation reactions, have suffered a block in a reaction near the end of the cAMP-induced pathway leading to growth regulation. In that case, the particular phosphorylation processes lost by the latter type of mutant may be directly related to the biochemistry of growth regulation (67a).

It has been suggested that the action of cAMP on transformed cells involves change in the exposure of particular genetic regions so as to make them accessible to regulatory signals transferred from information gathered at the site of the membrane receptors (171, 220, 221). In partial test of this hypothesis, experiments have been carried out studying the effects of reverse transformation on the sensitivity of the DNA of CHO cells to degradation by nucleases. These studies have demonstrated that in normal cells the amount of hydrolysis of chromatin preparations subjected to the action of nuclease I is markedly greater than that of transformed cells. Addition of cAMP has virtually no effect on normal fibroblasts, but the sensitivity of the DNA in the transformed CHO cell is restored to that of the normal cell by the addition of cAMP (Schonberg et al, in preparation). It is hoped that the specific chromosomal regions which are so affected will be identified. Experiments with azacytidine, which can remove methyl groups from cytosine moieties of DNA and permit new gene expression (119), have shown that demethylation can also mimic some of the effects of cAMP on the transformed CHO cells (Puck et al, unpublished data).

Chromosomal instability is another characteristic differentiating malignant from normal cells. It has been shown that karyotype instability in the CHO cell can be enormously increased by adding extremely small amounts of cytoskeletal disrupting agents like colchicine. Concentrations too small to block the cell in mitosis introduced large amounts of nondisjunction so that highly variable chromosomal constitutions were obtained (35). Thus, cells with damaged cytoskeletons may be more susceptible to nondisjunctional processes and, therefore, to production of aberrant karyotype with distorted metabolic balances. The existence of such karyotype instability in cancer cells may then be due to damage to their cytoskeletons. This situation seems capable of explaining why cancer cells exhibit rapid development

of resistance to therapeutic agents. The variability in chromosomal constitution makes possible continuous evolutionary selection of forms with increased resistance to the growth-inhibiting therapeutic agent.

FURTHER APPLICATIONS TO MEDICINE

A number of implications for medicine have already been indicated in preceding pages. Space limitations make possible consideration of only a few of the many topics in this connection. The development of monoclonal antibodies offers enormous new possibilities in both medical diagnosis and treatment. The new understanding now developing of the actions governing gene expression including the role of peptide and steroid hormones, changes in gene methylation, the interactions of members of multigene families, the actions of transposable genetic elements, and the metabolic changes affected by protein phosphorylation and dephosphorylation promise fundamental new understanding of the molecular biology of normal and abnormal developmental processes. These will undoubtedly have profound implications for human health and disease.

What are the characteristics of a disease due to a somatic cell mutation? It would appear that a necessary feature of such a pathogenesis is that a mutation affecting a single somatic cell must be amplified to the point where pathology is produced. Thus, the affected cell must be able to multiply after the mutagenic process, so as to produce a clone sufficiently large that the common genetic defect of its members can exert a pathological effect on the organism as a whole. One class of diseases which fulfill these requirements are the genetic diseases which display mosaicism (7). Here a single gene or chromosomal mutation arises after cell division has been initiated in the embryo, so that only a restricted clone of cells is affected. If these cells do not include the germ cells, the pathology will be limited to the individual in which the original mutation took place. It is conceivable that many congenital birth defects for which no heritable concommitant is demonstrable may share these characteristics.

Another example of somatic cell genetic disease is cancer, at least in some of its forms. A variety of malignancies have been shown to have characteristic chromosomal abnormalities like those mentioned above. A gene or chromosomal mutation at a particular site, which can override the normal growth regulatory mechanism of specific tissue cells, can lead to unchecked multiplication of the mutant cell and the formation of a clone which can threaten the life of the organism.

Another set of diseases that may well qualify as somatic cell diseases are the autoimmune conditions (70, 72). Normally, leukocytes are produced which are programmed to elicit antibodies for almost any conceivable anti-

genic determinant. Contact with the specific antigen stimulates these cells to divide and to manufacture large quantities of their particular antibody species. However, those cells programmed to yield antibodies that would react with the organism's own tissues are normally prevented from initiating such an induction by a genetic mechanism that as yet has not been elucidated. A mutation in a single cell at this locus could remove this block and permit reproduction of these defective cells. The resulting clone would manufacture large quantities of the forbidden antibodies, which in turn attack the body's tissues. The very large number of autoimmune disease conditions in man gives these considerations great importance.

A particularly interesting situation has been afforded by a new development with respect to the disease scleroderma. This condition has been shown to involve autoimmune antibody production against chromosomal centromeres of the somatic cells (180). Here then is what may well be a somatic-genetic defect that causes antibodies to be produced that attack the somatic cell chromosomes themselves, so that a two-fold set of somatic cell genetic events may be involved.

Somatic cell genetic approaches now show promise of enormously important application in the detection of mutagenic and carcinogenic agents in the environment. Ames and his collaborators (3) first introduced effective methods of screening for environmental mutagens, using bacteria as the test organisms. This advance, while extremely important, still has serious limitations for detecting actions affecting the human genome. This limitation arises because the Ames test is highly efficient for detection of point mutations, but much less efficient in the detection of deletions and nondisjunctional events that would cause death of the test cell. Whenever the test gene is contained on a chromosome that also contains other genes necessary for reproduction of the test cell, this situation occurs. We have demonstrated that incorporation of single human chromosomes in the CHO cell can yield a stable hybrid in which large numbers of convenient genetic markers located on the human chromosome can be utilized for the detection of mutagenic action (284). Since the human chromosome incorporated in the hybrid is not needed for cell reproduction, such events can be accurately scored so that a much wider range of genetic insults from environmental agents can be examined. The very large contribution of human aneuploidy of both partial and whole chromosomes to human genetic disease makes these developments appear to be important for the screening of environmental mutagens.

The methods outlined here demonstrate how, at least in principle, the methods of somatic cell genetics and genetic biochemistry could be used to delineate biochemical chains underlying almost any metabolic process. The considerations outlined in this paper indicate how a genetic-biochemical

attack, involving preparation and isolation of mutants with a specific phenotype and their subsequent genetic and biochemical analysis, should make possible elucidation of the biochemical pathways and their regulatory mechanisms for an extremely wide variety of differentiation and developmental processes. It can hardly be doubted that these studies will have a profound effect in elucidating the molecular basis of large areas of human disease which are now obscure, and help in designing new approaches to diagnosis, prevention, and treatment of human disease.

Finally, mention should be made of the recently developed technology whereby appropriate human DNA sequences can be incorporated into microorganisms under conditions such that these genes are expressed so that fermentative preparation of a variety of human proteins, such as specific antibodies (137), hormones, and other agents, can be prepared in large quantities and at small cost.

SUMMARY AND CONCLUSIONS

Somatic cell genetics is a new genetic discipline applicable to multicellular eukaryotic organisms, which bypasses the need for sexual mating and permits genetic analysis and genetic biochemical study of somatic cells. It yields much more rapid genetic analysis than is possible by means of standard mating procedures and makes possible exploration at molecular levels of genetic and genetic biochemical phenomena. It is especially useful in analysis of human genetic processes and study of differentiation and developmental phenomena.

The basic somatic cell genetic approaches which are strongly rooted in the microbial genetic procedures involve the following steps:

1. Development of stable clonal populations from any human or animal subject.
2. Characterization of the karyotype.
3. Construction of single cell survival curves for cell cultures of interest when treated with physical, chemical, or biological agents affecting cell reproduction.
4. Selection of mutants with respect to characteristics of interest including regulatory actions.
5. Complementation analysis to determine dominance-recessive relationships and to assign mutants to appropriate complementation groups.
6. Reversion analysis to differentiate between simple and complex mutations.
7. Biochemical analysis to determine the specific metabolic block in each complementation group and to establish the individual steps of the normal biochemical pathway.

8. Preparation of appropriate hybrids so as to identify the human chromosomes carrying the genetic determinants of interest.
9. Mapping of the appropriate genes.
10. Determination of the existence of gene families and of the regulatory mechanisms governing expression of the loci in question.
11. Preparation of DNA probes for specific regions of the genome.

These new approaches promise specific advances in diagnosis and treatment of a wide variety of human diseases of both germ cell genetic and somatic cell genetic nature. They should also have important applications in preventive medicine, but their greatest application to human health should come about as a result of the new understanding of the molecular nature of normal differentiation and developmental processes and their aberrations in disease situations.

Science in our time has given man the means to destroy the world by weapons of incredible devastation, but it is now also providing promise for new understanding of man's biological nature and fulfillment of his health needs in a fashion previously undreamed of. The message of these new possibilities, to which genetic science is contributing so effectively, must be made clear so that enlightened choices can be made by mankind in the coming decades.

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