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Chromosome Mapping of Connective Tissue Protein Genes

Robert L. Church

W. Alton Jones Cell Science Center, Lake Placid, New York

I. Introduction

Recent advances in the fields of cell culture and somatic cell genetics have made possible the attempt to identify and isolate the individual genetic elements that combine to form normal connective tissues in mammals. At this point, the total number of structural genes and regulator elements responsible for producing various connective tissues in humans is unknown. However, a few genetic loci have been mapped which involve presumptive interactions of connective tissue, mainly in the laboratory mouse (Green, 1975).

Many different chromosomal syndromes appear to involve the proteins in the extracellular matrix, both as primary disorders or as particular aspects of systemic disorders (McKusick, 1972). Most of these disorders have been linked to errors in the synthesis or posttransla-

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tional modification of the various collagens (Minor, 1980) or proteoglycans (McKusick, 1972), which are the most abundant elements in the extracellular matrix. Investigations concerning the identification and study of genetic elements responsible for production of the extracellular matrix are very important for several reasons. First, there exists a very large population of individuals who are affected by problems concerning the extracellular matrix, from damaged tendons or cartilage in athletes to the resultant manifestations of many birth defects. Second, once genes responsible for the production of specific elements of connective tissue are identified, the work of identifying exact lesions that cause connective tissue diseases can begin. Third, manipulation of defective connective tissue genes can be carried out to correct or "cure" the disease; finally, studies of extracellular matrix genes can supply information about development and maturation of connective tissues during embryogenesis and their effects on induction of tissue development.

The main thrust of this article will be to introduce and describe the techniques now available for investigating the products of specific chromosomes, particularly human chromosomes, as expressed in somatic cell hybrids. Very little experimental evidence exists at present that utilizes these techniques for investigations of connective tissues. This evidence will be presented, mainly as a working example and not as finished and unambiguous data.

II. History of Somatic Cell Hybridization

The appearance of multinucleated or fused cells *in vivo* is a relatively common occurrence. For instance, in diseased states (e.g., tuberculosis, variola, or vaccinia) polykaryons have been observed since the early nineteenth century. Obvious cases of normal *in vivo* cell fusion involve the production of myotubes from myoblasts (observed also *in vitro*), osteoclast production, or fertilization (Ringertz and Savage, 1976). Experimental *in vivo* hybrid production was demonstrated by Mintz and Baker (1967), who showed that chimeric mice, produced by mixing blastomeres of two separate strains of mice (for instance black-coated and white-coated mice) and reimplanting the resulting "mixed" blastocyst into a pseudopregnant foster mother, had muscle tissue (myotubes) formed from both parents (Mintz and Baker, 1967; Mintz, 1971; Carlsson *et al.*, 1974).

The first *in vitro* evidence of somatic cell hybridization was reported by Barski *et al.* (1960) who isolated a line of hybrid cells containing the entire chromosomal complement of two separate mouse sarcoma-pro-

ducing cell lines. These observations were confirmed shortly thereafter by Sorieul and Ephrussi (1961) and Gershon and Sachs (1963). The production and isolation of such hybrid cells was a laborious task since the frequency of spontaneous fusion is very low (less than 1 in 200,000 cells). Also, unless the hybrid has different growth characteristics from the parental cells (hybrid vigor), it is almost impossible to separate from either parent. In 1964, John Littlefield reported a technique which, with few exceptions, solved the problem of hybrid selection (Littlefield, 1964). This technique, which has been greatly expanded and altered since, involved utilizing parental cells having a particular growth mutation such that the heterohybrid cells survived and the unhybridized parents, or homohybrids, died in the selective medium. A more detailed discussion of these procedures follows in Section III,A.

The frequency of hybridization events was increased dramatically (up to 1000-fold) by the use of agents, particularly inactivated Sendai virus, that induce the fusion of mammalian cells (Harris and Watkins, 1965; Okada and Murayama, 1965; Ephrussi and Weiss, 1965; and Yerganian and Nell, 1966). Many other chemicals such as polyethylene glycol or lysolecithin have also been used to increase the number of cell fusion events for any particular experiment (for a discussion of nonviral fusogens, see Ringertz and Savage, 1976).

Finally, Weiss and Green (1967) discovered that a preferential loss of human chromosomes occurred in mouse–human somatic cell hybrids and suggested that this phenomenon could be used to assign genes for particular functions to human chromosomes. This procedure has been used by many laboratories to make assignments for at least 175 genes on all 22 autosomes and the X chromosome (Shows and McAlpine, 1979). Another major breakthrough in chromosome mapping came with procedures for accurately identifying particular chromosomes in hybrids. Caspersson *et al.* (1970) showed that quinacrine mustard stained chromosomes with a reproducible and specific fluorescent banding pattern unique for each chromosome. Several nonfluorescent staining procedures utilizing Giemsa (Schnedl, 1974a; Sumner *et al.*, 1971) have since been introduced, all of which have made chromosome identification a relatively simple procedure.

Newer methods of hybrid production such as microcell and isolated chromosome-mediated hybridization (Ege and Ringertz, 1974, McBride and Ozer, 1973; Sundar-Raj *et al.*, 1977; Willecke and Ruddle, 1975) have simplified and speeded up the process of chromosome identification and gene mapping; these techniques allow for a very limited amount of genetic material to be transferred from one parent onto the genetic background of a second parent cell.

III. Techniques Used in Somatic Cell Hybridization

A. Selection Systems

In order to produce and isolate somatic cell hybrids in the numbers and with the homogeneity required for gene assignment or regulation, one must be able to isolate quickly true hybrids (cells having genetic material contributed by both parents) from unfused parent cells or from fused cells which contain nuclei from only one parental type (homokaryons). Littlefield (1964) utilized a system initially devised by Szybalski *et al.* (1962) called HAT (hypoxanthine–aminopterin–thymidine) selection. This system combines two separate mutant cell parents, one thymidine-kinase deficient (TK^-) and the other hypoxanthine-guanine phosphoribosyl transferase deficient ($HGPRT^-$). Since this system has been widely used in cell hybridization, a description of the procedures used to generate such mutants will be given. These procedures, with minor modifications, are applicable to most other mutant selection systems used in generating somatic cell hybrids.

In general, drug resistance in animal cells can be obtained spontaneously simply by growing the cells in the selective agent. However, mutants can be generated much faster if the cells are initially treated with chemical mutagens or X-irradation (Chu and Malling, 1968; Bridges *et al.*, 1970; Sato *et al.*, 1972; Hsie *et al.*, 1975; Friedrich and Coffino, 1977). The most popular mammalian cell mutagens include ethyl methanesulfonate (EMS), methyl methanesulfonate (MMS), and nitrosoguanidine (NG), all of which are DNA-alkylating agents that introduce unstable "hot spots" into the DNA molecule. The mutagen of choice in our laboratory is EMS and is usually administered to cells in a single dose of 50–800 μg/ml in culture medium, depending on the cell line used. Once the cells have been treated, one or two days' growth in normal medium is carried out to allow multiple expression of the mutant phenotypes, after which the cells are selected for conditionally lethal growth mutations.

Mutagen-treated cells grown in the purine analogs 8-azaguanine (8-Azg) or 6-thioguanine (6-Tg) produce active HGPRT. HGPRT converts these analogs into defective nucleotides (Fig. 1) which results in cell death. However, a small percentage of these cells have lesions in the structural gene for HGPRT (Beaudet *et al.*, 1973; Wahl *et al.*, 1975). Therefore, they do not metabolize 8-Azg or 6-Tg and will grow "normally" in culture containing these analogs. Eventually, a cloned cell line is obtained that cannot utilize the "salvage pathway" of nucleotide production and must synthesize purine nucleotides *de novo*. This line is

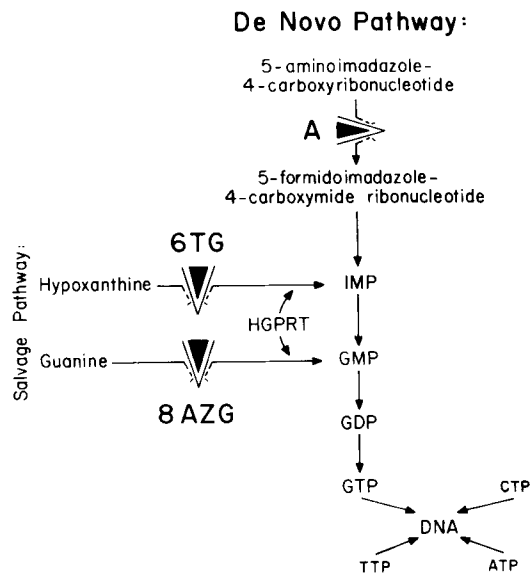

FIG. 1. Pathway of purine biosynthesis demonstrating the mutant selective action of 6-thioguanine (6Tg) and 8-azaguanine (8Azg), selecting for HGPRT$^-$ mutants. The blocking action of aminopterin (A) in the *de novo* pathway is shown.

usually designated as HGPRT$^-$. Final concentrations of 8-Azg required to obtain a stable mutant range from 2 to 500 μg/ml and of 6-Tg from 5 to 10 μg/ml, depending on the cell line used. In general, both purine analogs are used in tandem to obtain an HGPRT$^-$ mutant. Use of only one analog can often result in a cell membrane transport mutant rather than an HGPRT mutant (Harris and Whitmore, 1974). HGPRT$^-$ human cell lines can be obtained simply by using fibroblasts from patients having the Lesch–Nyhan syndrome, which is an X-linked disorder characterized by a stable deficiency of HGPRT.

Thymidine-kinase (TK) deficient mutants are selected by much the same procedure as just outlined except that bromodeoxyuridine (BUdR) is the selective analog used. BUdR blocks the salvage pathway of thymidine biosynthesis at the TK step (Fig. 2). Both mutants can survive normally either in analog-containing medium or in normal medium because the *de novo* pathways for purine or thymidine synthesis are intact.

Classically, these two mutant cell lines, one TK$^-$–HGPRT$^+$ and one TK$^+$–HGPRT$^-$, are fused and hybrids are selected in HAT medium. Cells grown in medium containing aminopterin [a potent inhibitor of

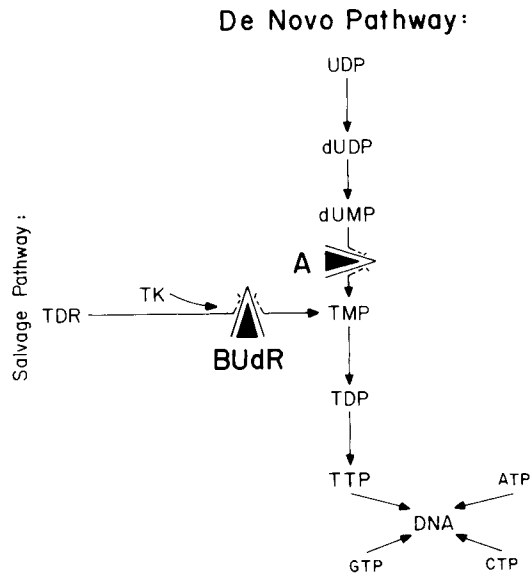

FIG. 2. Pathway of thymidine biosynthesis demonstrating the mutant selective action of bromodeoxyuridine (BUdR) on the salvage pathway, selecting for TK^- mutants. Aminopterin (A) blocks the *de novo* pathway at the step indicated.

dihydrofolate reductase, which is essential for *de novo* synthesis of both purines and pyrimidines (Fig. 1 and 2)] can survive if hypoxanthine and thymidine are present in the medium *if* the salvage pathways are intact. Any cell deficient in HGPRT or TK will die in the presence of aminopterin. However, if $HGPRT^-$–TK^+ and TK^-–$HGPRT^+$ cells are fused, the hybrid cells will live in the presence of aminopterin (as HAT medium) because the cell obtains HGPRT from one parent and TK from the other. This particular selective system works well if both parents have conditionally lethal mutations. As mentioned earlier, human Lesch–Nyhan cells are natural mutants for HGPRT deficiency and can readily be fused and hybrids selected. For human gene mapping studies where the human parent must have a diploid karyotype and be as normal as possible, isolation of mutants is not practical. However, diploid human fibroblasts (or other diploid human cells) have a finite life in cell culture and thus hybrids between rodent mutant cells and human diploid cells can be made using a "half-HAT" selection; the mouse parent is killed by the selection medium, and the human parent is quickly overgrown by the hybrids, which usually have a transformed phenotype including rapid growth. Also, cells such as primary lymphocytes, which do not attach and grow on tissue cul-

ture surfaces, can be used to obtain hybrids. Recently, the addition of ouabain to HAT medium (HATO) has greatly aided the selection of rodent–human hybrids. Ouabain blocks the Na^+,K^+–activated ATPase in cell membranes, inhibiting the growth of cells in culture (Mayhew and Levinson, 1968; McDonald *et al.*, 1972). It has been demonstrated (Baker *et al.*, 1974; Mankovitz *et al.*, 1973) that rodent cells such as mouse fibroblasts have a natural 1000-fold greater resistance to ouabain than human diploid cells. Since this resistance is codominant, mouse cells and mouse–human hybrid cells are not killed by ouabain. However, unhybridized human cells are killed. Thus, rodent–human hybrid selection can readily be made using HATO medium. Since the introduction of the HAT hybrid selection system, many more selective systems have been developed which, in general, take advantage of altered growth conditions in induced mutants. At least 20 hybrid selective systems now exist, many of which also select specifically for particular human chromosomes (Table I). These selection systems are valuable for use in gene mapping: If a human function invariably associates itself with the selected phenotype (such as HGPRT) or is lost by backselection (growth in 8-Azg and 6-Tg medium) causing the loss of the selected phenotype, then this human function has a high probability of being on the same chromosome. For instance, the TK structural gene was assigned several years ago to human chromosome 17 (Miller *et al.*, 1971; Boone *et al.*, 1972). Utilizing the growth of hybrids from TK^- mouse parents fused with human skin fibroblasts in the selective medium HAT, galactokinase activity and the production of human skin procollagen type I have been shown to have a syntenic relationship with TK. Therefore, the genes for these characteristics have been assigned to human chromosome 17, along with TK (Elsevier *et al.*, 1974; Sundar-Raj *et al.*, 1977). Many other systems are being devised for the selection of human chromosomes in rodent–human hybrids to aid further in human gene mapping (Siminovitch, 1976; Creagen and Ruddle, 1977).

B. Cell Fusion

As mentioned earlier, *in vivo* cell fusion is a normal and integral occurrence in most higher organisms, but it is normally restricted to certain tissues, e.g., skeletal muscle and osteoclasts. Theoretically, *in vitro* cell fusion can take place between *any* cells in any proportions. In general, the dynamics of cell fusion initially involve a close association of the adjacent cell membranes from two cells, usually induced by viral or chemical means (agglutination). Subsequently, the cell membranes

TABLE I

SELECTIVE SYSTEMS USED WITH RODENT–HUMAN HYBRIDS

Genetic defect	Selective medium	Human chromosome retained	Rodent mutant selection	References
Uridine kinase$^-$	Adenosine–uridine	1	5-Fluorouridine	Medrano and Green (1974)
LETS$^-$[a]	Collagen-coated plastic petri dish	8?	?	Owerbach *et al.* (1978)
Adenosine kinase$^-$	Alanosine	10	6-Methylthiopurine ribonucleotide	Bennet *et al.* (1966); Chan and Creagan (1976)
Adenine phosphoribosyltransferase$^-$	Alanosine–adenine	16	2,6-Diaminopurine or 2-fluoroadenine	Kusano *et al.* (1971); Tischfield and Ruddle (1974)
Galactokinase$^-$	Medium containing galactose	17	2-Deoxygalactose	Thirion *et al.* (1976)
Thymidine kinase$^-$	HAT	17	BUdR	Szybalski *et al.* (1962); Littlefield (1964)
Hypoxanthine phosphoribosyltransferase$^-$	HAT	X	8-Azg or 6Tg	Szybalski *et al.* (1962); Littlefield (1964)

[a] Large, external, transformation-sensitive.

appear to "coalesce" with one another, forming cytoplasmic bridges between the two cells, followed by breakdown or rearrangement of intervening membranes. This results in one cell having two nuclei (polykaryon) and complete cytoplasm from both cells (for a more detailed discourse of the dynamics of fusion, see Ringertz and Savage, 1976). If the two cells being fused are identical, the resulting multinucleate cell is called a homokaryon. If the cells are nonidentical, the cell is termed a heterokaryon (only this type of cell is termed a hybrid).

1. Virus-Mediated Cell Fusion

Harris and Watkins (1965) first demonstrated the practicality of using virus-mediated cell fusion for obtaining multinucleated cells from intraspecific or interspecific parental cells. The most widely used virus for cell fusion is inactivated Sendai virus (Okada, 1958; Compans *et al.*, 1964; Giles and Ruddle, 1973; Poste, 1972). Inactivation of the virus is carried out using β-propiolactone or UV light, which ensures that the virus acts only as an agglutinating agent and does not replicate. Although some objections have been raised to the use of a viral agent, even inactivated, to initiate cell fusion, Sendai virus remains the most common agent for hybrid production. Sendai virus is in the class of RNA-containing viruses generally called paramyxoviruses, and subclassed as a parainfluenza type (Okada, 1958; Compans *et al.*, 1964). Many other viruses, both DNA- and RNA-containing types, have been observed to induce cell fusion (Table II). The primary determinants for the ability to cause fusion between cells apparently reside

TABLE II

Viruses which Induce Cell Fusion

Virus	References
RNA viruses	
Coronavirus	
Avian infectious bronchitis	Akers and Cunningham (1968)
Oncornavirus	
Rous sarcoma	Moses and Kohn (1963)
Paramyxovirus	
Sendai	Okada (1958)
Mumps	Henle *et al.* (1954)
Newcastle disease	Kohn (1965)
DNA viruses	
Poxvirus	Appleyard *et al.* (1962)
Herpesvirus	Hoggan and Roizman (1959)

in the receptors on the mammalian cell membrane, and the ability of the virus to "stick" to these receptors, much the same way erythrocytes are agglutinated and caused to lyse (Yanovsky and Loyter, 1972). However, it has been proposed that paramyxoviruses contain a specific fusion factor (Guggenheim *et al.,* 1968; Okada, 1969; Kohn, 1965) which is contained in the phospholipid of the viral envelope.

Often these viruses display a specificity of fusion, depending on the host cells from which the active virus is generated. This specificity is due, in part, to the nature of the receptor sites on the virus envelope, which come from the host cell plasma membrane as the finished viral particles are extruded (Howe *et al.,* 1967; Blough, 1964). Poste (1970) has generalized the conditions under which fusion is induced in various cells. These conditions are summarized as follows: established cells fuse better than primary or secondary passage cells, malignant cells fuse better than nonmalignant cells, and younger cells fuse better than older (senescent) cells in relation to passage number.

Two general methods for virus-mediated cell fusion have been reported. These differ only in that fusion occurs using cells in suspension (Watkins, 1971; Rao and Johnson, 1972; Harris and Watkins, 1965) or as monolayer culture (Hitchcock, 1971; Klebe *et al.,* 1970). Giles and Ruddle (1973) have compiled detailed recipes for both fusion procedures, including the "do's" and "don'ts." However, the optimum cell number to use or the amount of virus to be added usually must be calculated for each new cell type in order to produce the greatest number of hybrids.

2. Nonviral Cell Fusion Agents

There are many chemical agents which have been shown to induce cell fusion. Most, if not all, of these agents act on the cell surface and cause cell aggregation, which leads to fusion. Perhaps the simplest method for inducing cell fusion is to alter the calcium ion concentration in the culture medium (Toister and Loyter, 1971, 1973; Keller and Melchers, 1973). Since calcium ions are important in cell attachment to most surfaces, as well as in Sendai virus-mediated fusion, this effect may be an important general requirement for all cell fusion.

Lipids and lipid-related compounds have been used to induce fusion in a variety of cell types. Lysolecithin has been the best studied of this group (Poole *et al.,* 1970; Croce *et al.,* 1971). However, this molecule is extremely toxic to living cells (Ahkong *et al.,* 1972) and thus its use is limited. Other lipid-related substances such as fatty acids or glycerol

derivatives have been used as fusogens. In this group, glycerol monooleate has proved to be an effective fusion agent, inducing hybrid formation four to seven times greater than spontaneous fusion (Cramp and Lucy, 1974). Recently, Mukherjee *et al.* (1978) have utilized entrapment of chromosomes by phospholipid vesicles (lipochromosomes) to allow transfer of genes into intact cells. It is unclear whether this process involves actual fusion of the lipochromosome to the intact cell or phagocytosis of the particle. Papahadjopoulas *et al.* (1973, 1974) have utilized liposomes (small lipid vesicles) to achieve a relatively high amount of cell fusion.

Perhaps one of the best agents for routine cell fusion is polyethylene glycol (PEG), which was first used to fuse plant cells (Kao and Michayluk, 1974). This chemical avoids the use of virus and is extremely easy to handle. Several different molecular weights of PEG have been used, ranging from 6K to 7.5K (Pontecorvo, 1975; Davidson and Gerald, 1976; Steplewski *et al.*, 1976; Hansen and Stadler, 1977), to about 1K (Gefter *et al.*, 1977; Hales, 1977). The structure of PEG is shown in Fig. 3; the differences in molecular weight are achieved by additions of the basic "unit" (in parentheses) to obtain the polymer size needed (the unit addition is about 150 to yield the 6K–7.5K polymer). This chemical is effective either with monolayer culture (see previous references) or in suspension culture (Hales, 1977). Others have used PEG in combination with dimethyl sulfoxide (DMSO) to achieve improved cell fusion (Norwood *et al.*, 1976). Because PEG is relatively toxic to animal cells, the optimum concentration and incubation times must be ascertained before its routine use.

The last and possibly most elegant method of fusing cells is to utilize microsurgery to initiate the fusion event (Diacumakos and Tatum, 1972; Diacumakos, 1973). This procedure involves fusing two isolated cells by mechanically attaching their cytoplasms together, which is usually followed by fusion of the cells. While this method is very specific in the products obtained, the high cost of obtaining a micromanipulator and highly trained personnel to carry out the procedure precludes its general use.

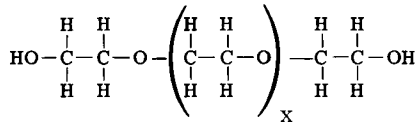

FIG. 3. Structure of polyethylene glycol (PEG). Additions of the basic unit, X, determine the polymer size.

C. *Hybrid Production and Cloning*

Using the previously discussed systems for fusion of cells and selecting hybrids, a "typical" hybridization experiment will be outlined and will reflect its use in human gene mapping. Figure 4 illustrates the steps involved in producing and selecting hybrids. The type of mouse parent used is important only if retention of a particular human chromosome in the hybrid is necessary for later gene assignment. Table I lists seven different mouse or Chinese hamster selection systems that will retain a particular human chromosome in the hybrid. For our purposes, mouse cell line LM(TK^-), which is negative for the production of TK, will be used as the mouse parent. Skin fibroblasts will be used as the human parent since this article deals with connective tissues. A tissue culture dish of human fibroblasts (HF) (the age of the donor seems not to have a greatly different effect on hybrid production) is grown to about half confluence. Mouse $LMTK^-$ cells are plated onto the human cells, and the mixed cells are allowed to grow for a few days until total cellular confluence is reached (i.e., until mouse and human cells are in close apposition to one another). Then a predetermined aliquot of β-propiolactone-inactivated Sendai virus in a small volume of serum-free culture medium (1 ml for a 100 mm tissue culture dish) is added to the mixed cell monolayer. The virus-treated culture is incubated in the cold (4°C) with occasional rocking (to continuously cover the cells with liquid) for about 1 hour. Without removing the virus preparation, prewarmed serum-containing medium is added, and the culture is kept in the CO_2 incubator (37°C) overnight. After the cells have recovered from the virus–cold treatment (about 1 day), they are treated with trypsin, resuspended in culture medium, diluted, and plated into selective medium (HAT medium). The cells are plated at low cell density (about 50,000 cells per plate) to allow optimal killing of the unhybridized parents and homokaryons. Plating these cells at high densities sometimes allows the cells to "cross-feed" one another and not suffer killing in the selective medium. Since the human parent is diploid and grows slowly at low cell density, the half-HAT selection procedure can be used. However, ouabain can be added to the HAT medium to achieve specific killing of the unhybridized human parent and human homokaryons. A concentration of 10^{-5} *M* ouabain in HAT medium will effectively kill the human parent cells while leaving the hybrid cells unaffected. Over a period of about a month, unhybridized cells or homokaryons are killed in the selective medium while the hybrid cells usually grow as tight colonies (as shown in the lower culture plate in Fig. 4). When the hybrid colonies reach a cell number of about

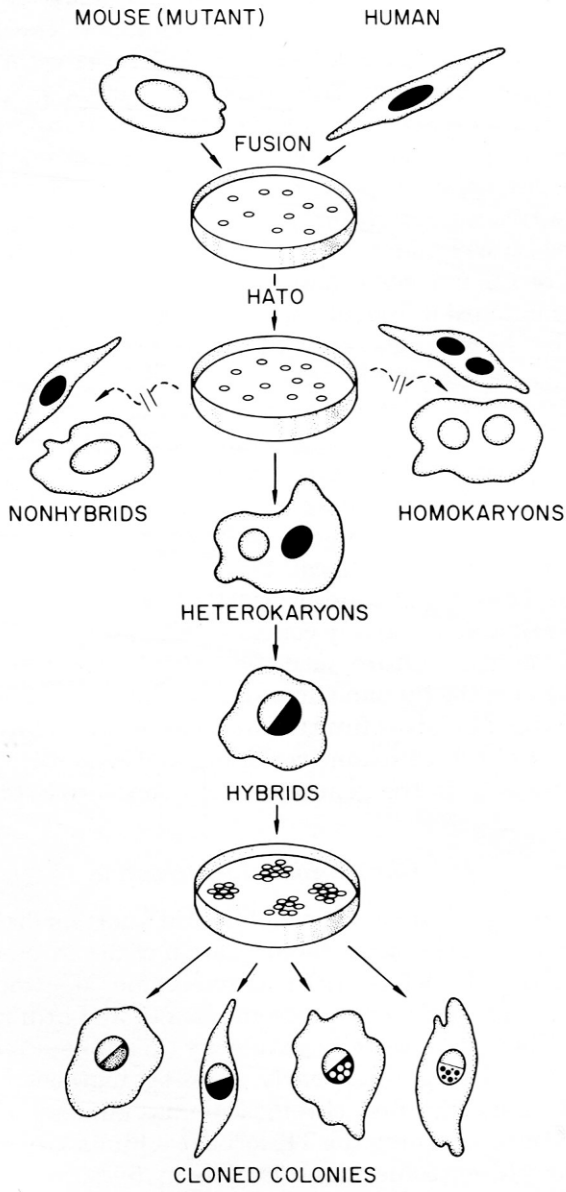

FIG. 4. Schematic representation of cell fusion and selection of hybrids. Nonhybrids and homokaryons are killed in the HATO selection medium. With human–mouse hybrids, the cloned colonies indicate a unique set of human chromosomes in each clone.

500–1000 cells, or a colony diameter of 5–7 mm, the colonies are picked out using a glass or stainless steel cylinder. These picked colonies are diluted with fresh HAT medium and replated at very low cell density (1–10 cells per dish). This step allows individual clones to grow up from one cell, thus achieving hybrid colonies having a homogeneous chromosome complement. The necessity of the cloning procedure resides in the unexplained phenomenon (for review, see Handmaker, 1973) of selective human chromosome loss in rodent–human hybrids, first observed by Weiss and Green (1967). This phenomenon is also the cornerstone of human gene mapping. Since this preferential loss of human chromosomes in rodent–human hybrids appears to be random (except for the chromosome retained due to the selection system used), we can isolate cloned hybrid lines having different combinations of human chromosomes against the intact mouse genetic background. The key step in this process is to obtain essentially all the combinations and permutations of human chromosomes in homogeneous clonal hybrid lines. Thus, by examining each line for the presence of particular human chromosomes and gene products, such as human collagen production, the gene products can be associated with specific human chromosomes. Once these lines are isolated, their human chromosome complement remains relatively constant. However, over a long period of time in continuous culture, or if the cells are frozen in liquid nitrogen and rethawed, the human chromosome complement of each hybrid can change, which involves further chromosome elimination. This fact results in the need for constant recloning and monitoring in order to maintain confidence in the genotype of the separate hybrids.

D. Chromosome Identification

Probably the most critical aspect of hybrid analysis for human gene mapping involves the accurate identification of the chromosomes present in each hybrid. Before 1970, chromosome identification relied mainly on measuring total chromosome length and arm ratios (Levan *et al.*, 1964). These data usually gave gene assignment only to groups of chromosomes. Using these early staining methods, Migeon and Miller (1968) made the first chromosome assignment using somatic cell hybridization, assigning the TK locus to a human group E chromosome. Human chromosome identification in human–mouse somatic cell hybrids was particularly difficult using regular chromosome stains because many mouse chromosomes in the hybrid were abnormal, having rearrangements and translocations which often made them in-

distinguishable from human chromosomes. These abnormal mouse chromosomes are common in most transformed mouse mutant cells used for hybridization.

1. Staining Methods

With the advent of new chromosome staining methods, the use of somatic cell hybridization for human gene mapping came of age. Caspersson and his colleagues first introduced the use of the fluorescent dye quinacrine (in several forms), which imparts specific and reproducible banding to chromosomes (Caspersson *et al.*, 1968, 1970, 1971) and allows them to be readily identified in rodent–human hybrids. However, this procedure involves the use of relatively expensive equipment and the fluorescence rapidly fades under the microscope. More or less permanently stained and banded chromosomes were successfully produced by different preparations of Giemsa stain (Arrighi and Hsu, 1971; Sumner *et al.*, 1971; Drets and Shaw, 1971; Schnedl, 1971; Seabright, 1971). The most successful and widely used procedure utilizing Giemsa stain is the method of Seabright (1971), which employs a pretreatment of the fixed chromosomes with trypsin, followed by Giemsa stain. This method of staining also produces very reproducible and specific bands (called G-bands) which readily allow identification of all of the human chromosomes. Several modifications of Seabright's procedure have been reported (see reviews by Schnedl, 1974b; Sanchez and Yunis, 1977). Figure 5 shows a diagrammatic representation of the normal human chromosome complement, showing the light and dark bands achieved by staining with quinacrine (Q-banding) or various treatments with Giemsa (G-banding). These specific bands serve to fingerprint each human chromosome. The banding patterns and numbering are those given in the report of the Paris Conference (1972). Other procedures which delineate human from mouse chromosomes in hybrids take advantage of the differential staining of centromeres and arms. Staining fixed and spread chromosomes with the fluorescent dye Hoechst 33258 (Hilwig and Gropp, 1972; Kucherlapati *et al.*, 1975) usually causes mouse, but not human, centromeres to fluoresce brightly (Fig. 6). A similar event occurs with Giemsa at an alkaline pH (Giemsa-11 staining; Bobrow *et al.*, 1972; Bobrow and Cross, 1974; Friend *et al.*, 1976a). Giemsa 11 stains mouse chromosomes magenta with light blue centromeres, and stains human chromosomes light blue with magenta centromeres. Therefore, this procedure can be used readily to identify human chromosomes in mouse–human hybrids

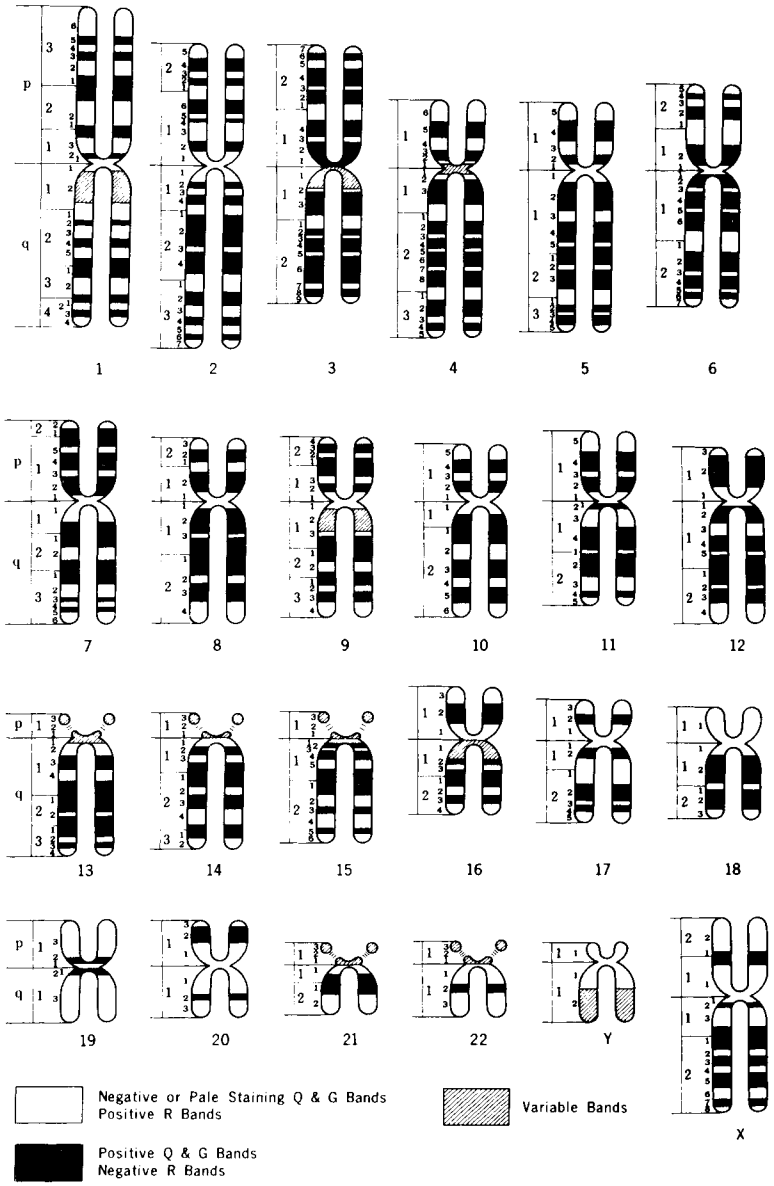

FIG. 5. Diagrammatic representation of human chromosomes as seen when stained for Q-,G-, and R-banding (Paris Report, 1972).

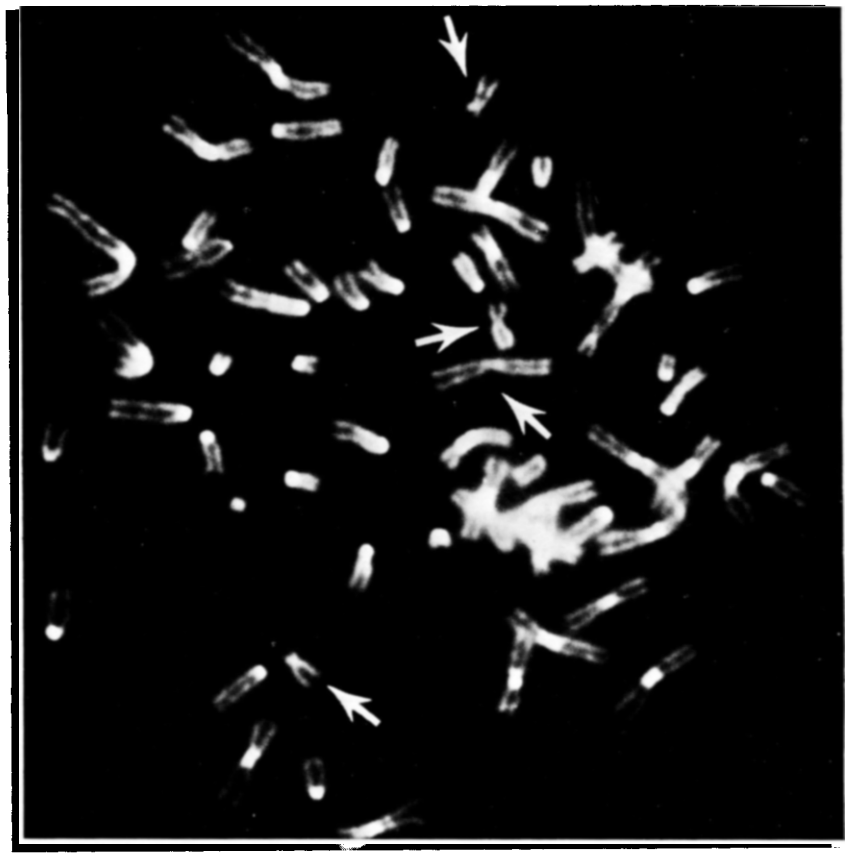

FIG. 6. Fluorescence photomicrograph of Hoechst 33258-stained metaphase chromosomes from a mouse–human somatic cell hybrid. Mouse centromeres are brightly stained while human chromosomes stain only slightly, with little or no centromeric staining. Some human chromosomes are identified by arrows.

(Friend *et al.*, 1976b). Figure 7 shows a Giemsa 11 stained mouse–human hybrid chromosome spread, having several human chromosomes (light arms).

Some procedures are often used in tandem, e.g., initial quinacrine fluorescent staining of hybrid metaphase spreads to visualize specific chromosomal bands (preserved with photography) followed by restaining the same spreads with Giemsa 11 to identify quickly human chro-

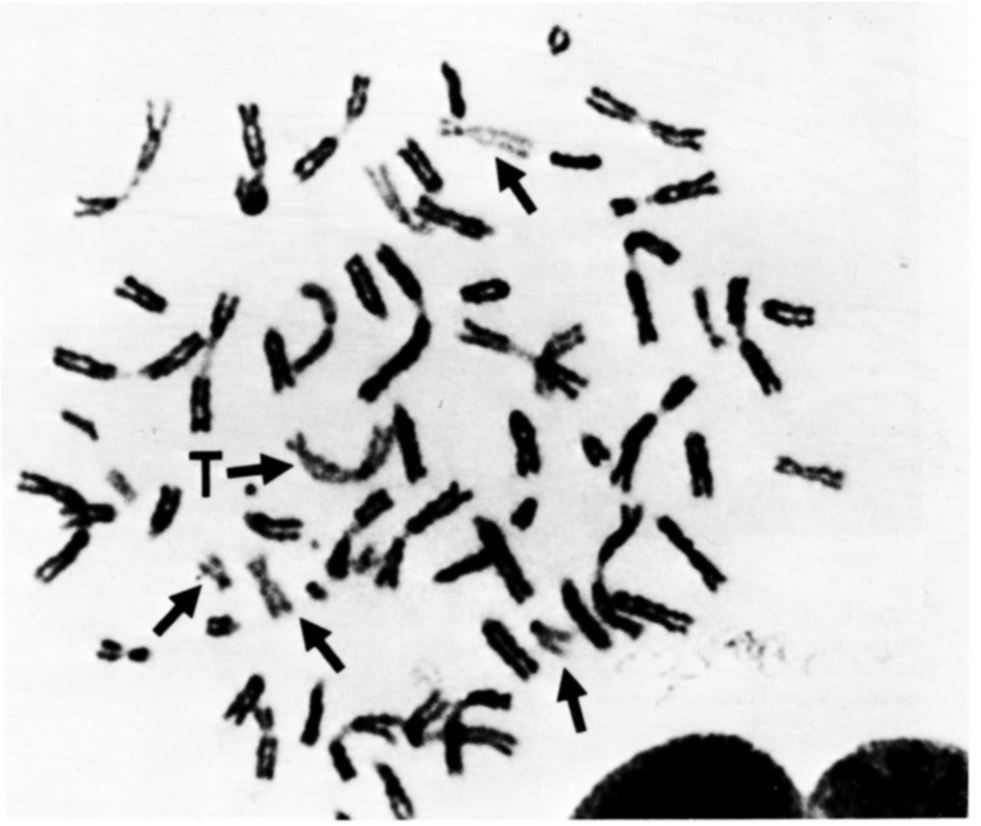

FIG. 7. Metaphase chromosome spread of mouse–human somatic cell hybrid as visualized by Giemsa 11 staining. Mouse chromosomes have darkly stained arms and lightly stained centromeres, while human chromosomes have light arms and light or dark stained centromeres. Arrows point out several human chromosomes plus a human translocation (T).

mosomes from mouse chromosomes. Such procedures now allow investigators to identify chromosomes quickly and accurately. They also have led to at least 120 genes being assigned to all 22 autosomes plus the X chromosome (Table III).

2. Isozyme Analysis

Most of the genes for which chromosome assignment has been made in Table III code for specific human enzymes, many of which exist in multiple molecular forms (called isozymes) differing mainly in charge but having the same catalytic reaction. Because of evolutionary differences between distantly related species, such as human and rodent, isozymes from these species usually differ in overall ionic charge and sometimes even in molecular weight. This difference in isozyme species has led to the development of electrophoretic methods for separating rodent and human isozymes. Because of the polymorphism of many isozymes, their identification in human-mouse somatic cell hybrids is usually accomplished by enzyme-staining procedures which yield either colored or fluorescent bands specific for the enzyme substrate used (for a detailed discussion and stain recipe for at least 100 different isozymes, see Harris and Hopkinson, 1976). Figures 8 and 9 show starch gel electrophoretic separations of two common isozymes in human–mouse somatic cell hybrids. Several other electrophoretic separation techniques exist including agarose gel electrophoresis, polyacrylamide gel electrophoresis, and isoelectric focusing. These techniques are often specific for those isozymes which do not give species separation using the more classic starch gel or cellulose acetate gel electrophoresis.

For some proteins, such as cell-surface antigens or virus receptor sites, immunofluorescence or other immunological procedures are used to identify the expression of the human protein (Jones *et al.,* 1975); Aden and Knowles, 1976). Also, several gene assignments have been made on the basis of sensitivity to toxins (diphtheria toxin sensitivity, chromosome 5), interferon production (chromosome 5), or virus integration sites (chromosomes 1, 7, and 17) (Creagan *et al.,* 1975; Tan *et al.,* 1973; Croce and Koprowski, 1974, 1975). Each of these gene assignments, once confirmed, can be used as an assay for the presence of the particular human chromosome to which the gene has been assigned. Again, these assays must always be used in conjunction with actual chromosome identification using the various staining procedures on metaphase spreads as outlined in Section III,D,1.

TABLE III

GENE ASSIGNMENTS FOR HUMAN CHROMOSOMES USING RODENT–HUMAN SOMATIC CELL HYBRIDS[a]

Symbol	Gene	Assignment[b]
Chromosome 1		
AdV-12-cms-1p	Adenovirus-12 chromosome modification site-1p	P
AdV-12-cms-1q	Adenovirus-12 chromosome modification site-1q	P
AK-2	Adenylate kinase-2	C
AMY-1	α-Amylase (salivary)	C
AMY-2	α-Amylase (pancreatic)	C
ENO-1	Enolase-1	C
FH	Fumarate hydratase	C
αFUC	α-L-Fucosidase	C
GDH	Glucose dehydrogenase	P
GUK-1	Guanylate kinase-1	C
PEP-C	Peptidase-C	C
PGM-1	Phosphoglucomutase-1	C
PGD	Phosphogluconate dehydrogenase	C
UGPP-1	Uridyl diphosphate glucose pyrophosphorylase-1	C
UMPK	Uridine monophosphate kinase	C
Chromosome 2		
ACP-1	Acid phosphatase-1	C
AHH	Aryl-hydrocarbon hydroxylase	P
Gal^+-Act	Galactose enzyme activator	P
IF-1	Interferon-1	P
IDH-S	Isocitrate dehydrogenase (soluble)	C
MDH-S	Malate dehydrogenase (soluble)	C
UGPP-2	Uridyl diphosphate glucose pyrophosphorylase-2	P
Chromosome 3		
$AF8ts^+$	Temperature sensitive complement	P
GPX-1	Glutathione peroxidase-1	P
HSV-1	Herpes simplex virus sensitivity (type I)	P
Chromosome 4		
PEP-S	Peptidase-S	C
PGM-2	Phosphoglucomutase-2	C
Chromosome 5		
ARS-B	Arylsulfatase-B	P
DTS	Diphtheria toxin sensitivity	C
HEX-B	Hexosaminidase-B	C
IF-2	Interferon-2	P
LEURS	Leucyl-tRNA synthetase	P
Chromosome 6		
GLO	Glyoxalase-I	C
GOT-M	Glutamic-oxaloacetic transaminase (mitochondrial)	P
ME-S	Malic enzyme (soluble)	C
Pg	Pepsinogen	P
PGM-3	Phosphoglucomutase-3	C
SA-6	Surface antigen-6	P
SOD-M	Superoxide dismutase (mitochondrial)	C
Chromosome 7		
Col-1	Collagen type I	P
βGUS	β-Glucuronidase	C

(*Continued*)

TABLE III (*Continued*)

Symbol	Gene	Assignment[b]
HADH	Hydroxyacyl-CoA dehydrogenase	P
MDH-M	Malate dehydrogenase, NAD (mitochondrial)	C
SV40-1	SV40-integration site	P
SA-7	Surface antigen-7	P
UP	Uridine phosphorylase	P
Chromosome 8		
GSR	Glutathione reductase	C
LETS	Large, external, transformation-sensitive protein	P
Chromosome 9		
ACON-S	Aconitase (soluble)	C
AK-1	Adenylate kinase-1	C
AK-3	Adenylate kinase-3	C
ASS	Argininosuccinate synthetase	P
Chromosome 10		
ADK	Adenosine kinase	P
EMP-130	External membrane protein-130	P
FUSE	Polykaryocytosis promoter	P
GOT-S	Glutamic-oxaloacetic transaminase (soluble)	C
GSAS	Glutamate α-semialdehyde synthetase	P
HK-1	Hexokinase-1	C
PP	Pyrophosphatase	C
Chromosome 11		
ACP-2	Acid phosphatase-2	C
Ala-1,2,3	Lethal antigens	P
ESA-4	Esterase-Ar^4	C
Hbβ	Hemoglobin β-chain	P
Hbγ	Hemoglobin γ-chain	P
Hbδ	Hemoglobin δ-chain	P
LDH-A	Lactate dehydrogenase-A	C
SA-1	Surface antigen-1	P
Chromosome 12		
CS	Citrate synthase	C
ENO-2	Enolase-2	C
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	C
LDH-B	Lactate dehydrogenase-B	C
PEP-B	Peptidase-B	C
SA-12	Surface antigen-12	P
SHMT	Serine hydroxymethyltransferase	P
TPI-1	Triosephosphate isomerase-1	C
TPI-2	Triosephosphate isomerase-2	P
Chromosome 13		
ESD	Esterase-D	C
Chromosome 14		
EMP-195	External membrane protein-195	P
NP	Nucleoside phosphorylase	C
TRPRS	Tryptophanyl-tRNA synthetase	C
Chromosome 15		
β-2m	β2-Microglobulin	C
HEX-A	Hexosaminidase-A	C
IDH-M	Isocitrate dehydrogenase (mitochondrial)	C

(*Continued*)

TABLE III (*Continued*)

Symbol	Gene	Assignment[b]
MAN-A	α-D-Mannosidase-A	P
MPI	Mannosephosphate isomerase	C
PK-M2	Pyruvate kinase (M2)	C
Chromosome 16		
APRT	Adenine phosphoribosyltransferase	C
Hbα	Hemoglobin α-chain	P
IFr	Interferon regulator	P
TK-M	Thymidine kinase (mitochondrial)	P
Chromosome 17		
AdV-5T	Adenovirus 5 T-antigen	P
AdV-12-cms-17	Adenovirus-12 chromosome modification site-17	P
Col-IS	Skin collagen type I	P
GALK	Galactokinase	C
GLU	α-Glucosidase	P
SA-17	Surface antigen-17	P
SV40-2	SV40-integration site 2	P
TK-S	Thymidine kinase (soluble)	C
Chromosome 18		
hCG	Human chorionic gonadotrophin	P
PEP-A	Peptidase-A	C
Chromosome 19		
E11s	Echo 11 sensitivity	P
GPI	Glucosephosphate isomerase	C
MAN-B	α-D-Mannosidase-B	C
PEP-D	Peptidase-D	C
PVS	Polio virus sensitivity	C
Chromosome 20		
ADA	Adenosine deaminase	C
DCE	Desmosterol-to-cholesterol enzyme	P
ITP	Inosine triphosphotase	C
Chromosome 21		
IFRec	Interferon receptor	P
GARS	Phosphoribosylglycinamide synthetase	P
SOD-S	Superoxide dismutase (soluble)	C
Chromosome 22		
ACON-M	Aconitase (mitochondrial)	C
ARS-A	Arylsulfatase-A	P
DIA-1	Diaphorase, NADH	P
X Chromosome		
αGAL	α-Galactosidase	C
G6PD	Glucose-6-phosphate dehydrogenase	C
HGPRT	Hypoxanthine-guanine phosphoribosyltransferase	C
OTC	Ornithine transcarbamylase	C
PGK	Phosphoglycerate kinase	C
PHK	Phosphorylase kinase	C
SAX-1,2,3	Surface antigens	P
TATr	Tyrosine aminotransferase regulator	C

[a] Nomenclature according to Shows and McAlpine (1979).

[b] "C" indicates that the gene assignment has been confirmed by at least two separate laboratories or two independent methods. "P" indicates provisional assignment. Inconsistent assignments are not included.

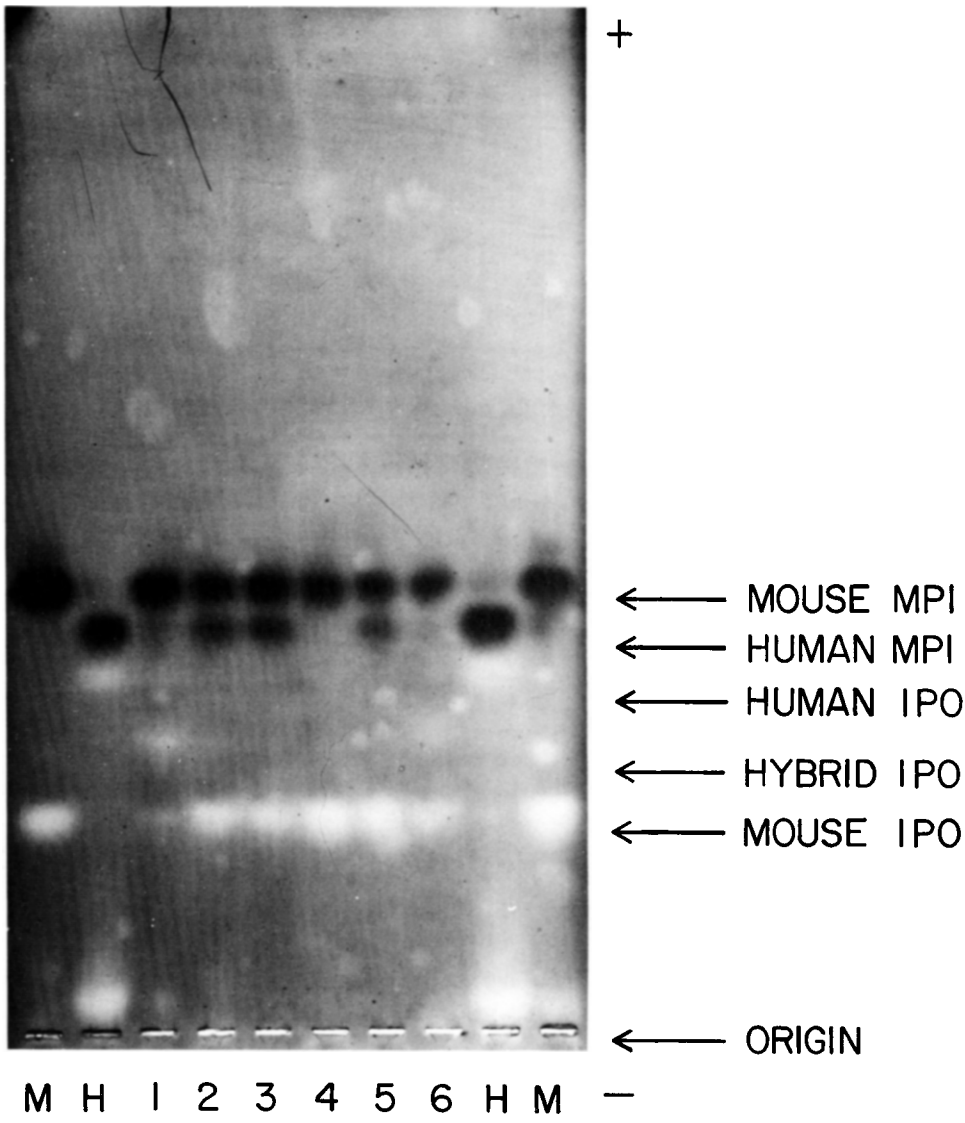

FIG. 8. Starch gel electrophoresis of mannosephosphate isomerase (MPI) (Nichols and Ruddle, 1973). Channels M are mouse A9 and LM cell standards. Channels H are human Hela and fetal skin fibroblast standards. Channels 1, 4, and 6 are mouse–human hybrids negative for human and mouse MPI. Channels 2, 3, and 5 are mouse–human hybrids positive for human and mouse MPI.

Also staining on this gel as light spots are indophenol oxidase (IPO), dimeric form, isozymes. Channels 1 and 6 are mouse–human hybrids positive for human IPO, while channels 2, 3, 4, and 5 are negative for human IPO.

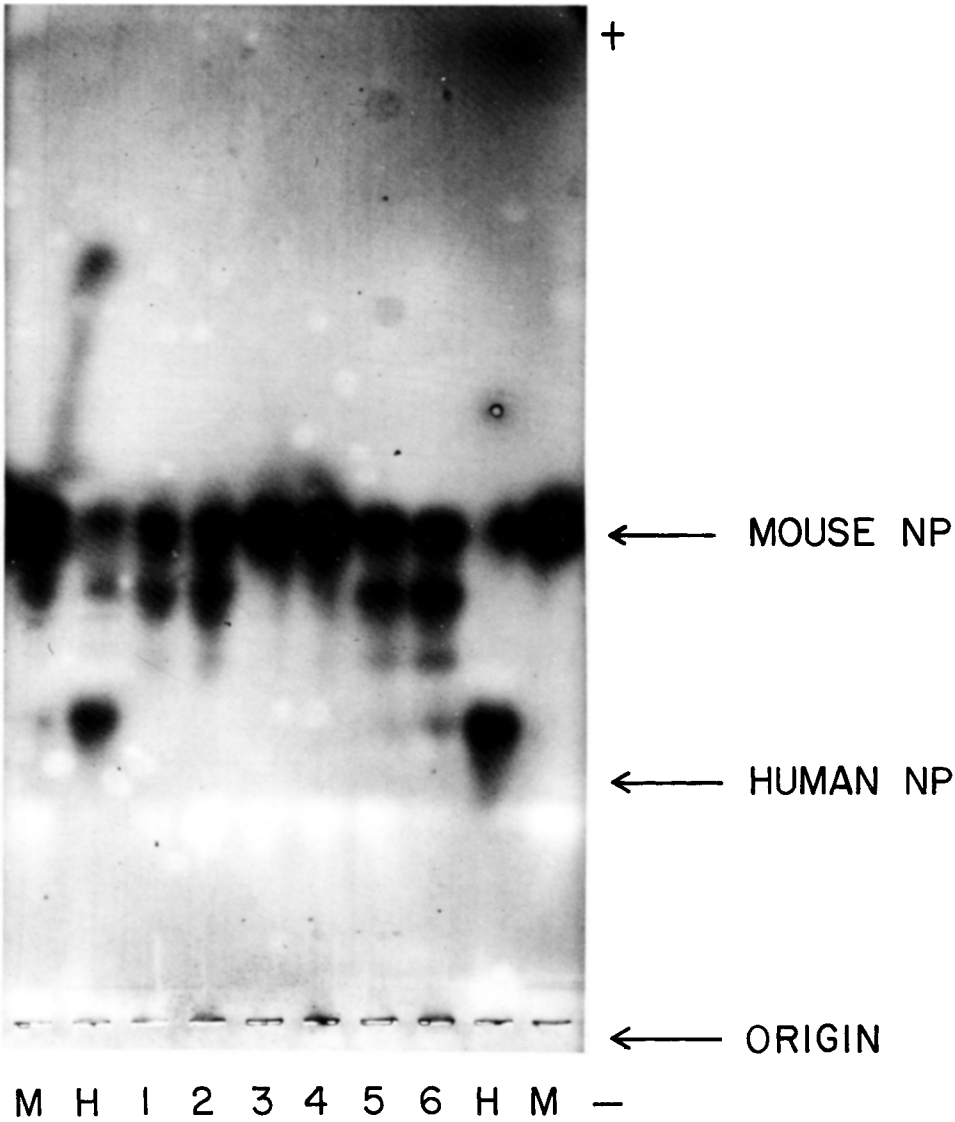

FIG. 9. Starch gel electrophoresis of nucleoside phosphorylase (NP). Channels M and H are mouse and human standards as described in Fig. 8. Channels 1, 2, 5, and 6 are mouse–human hybrids positive for both mouse and human NP. Channels 3 and 4 are mouse–human hybrids negative for human NP.

Mouse and human IPO also show up (light spots) but cannot be scored accurately in this particular gel.

E. New Methods for Producing Hybrids

During the past few years, procedures have been introduced for fusing cells and producing hybrids from cell fragments such as isolated nuclei, chromosomes, or enucleated cells (for a detailed treatment of this subject, see Ringertz and Savage, 1976). For use in human gene mapping, intact rodent cells can be fused with human nuclei (minicells), parts of nuclei (microcells), or isolated chromosomes. Each of these procedures has advantages over the normal hybridization procedures discussed earlier and, in general, can give results faster than conventional hybridization.

The production of minicells or microcells (which are simply nuclei or fragments of nuclei surrounded by nuclear membrane and a very small amount of cytoplasm and plasma membrane) depends on a phenomenon produced by the action of cytochalasin B on cultured cell. Cytochalasin B is a metabolic by-product obtained from the fungus *Helminthosporium dematioideum*. Carter (1967) first observed that cytochalasin B caused enucleation of mouse cells growing in culture. The exact mechanism of action of this metabolite is unknown (for review, see Poste, 1973), however, the treatment of cells in culture with cytochalasin B causes the nucleus to protrude from the cell, eventually leading to enucleation. This nucleus is surrounded by cytoplasmic membrane and is, in effect, still a living cell, now called a minicell (Ringertz and Savage, 1976). This minicell can be seeded onto a tissue culture dish where it will attach and metabolize. Minicells cannot replicate and cannot live for more than 2 days, but they will make viable hybrids.

1. Minicell Hybrids

Minicells can be produced in quantity by the application of a centrifugal force to the cytochalasin B-treated cells (Prescott *et al.*, 1972; Wright and Hayflick, 1972). When this procedure is used, the cells are grown on small plastic disks punched out of a tissue culture dish. The diameter of these disks is such that they fit exactly into a plastic or glass centrifuge tube. When the cells reach proper density, each disk is placed cell side down in a centrifuge tube containing cytochalasin B dissolved in culture medium (about 10 μg/ml cytochalasin B concentration). A plug is inserted over the disk to keep it from slipping during centrifugation, and the cell-coated disk is centrifuged to achieve enucleation. The speed and time of centrifugation and the cytochalsin B concentration are variable for each cell line used and must be determined beforehand. Also, some cell lines "stick" less readily than others, and an initial centrifugation without cytochalasin B must be

made to remove poorly adhered cells before minicell collection. Once the minicells are collected at the bottom of the centrifuge tube, they can be resuspended in culture medium and hybridization can be carried out normally (Fig. 10A), much the same as cell–cell hybridization (Fig. 4).

2. Microcell Hybrids

Microcells can be considered "miniature minicells"; because of the method of preparation, the amount of genetic material enclosed inside the nuclear and cytoplasmic membranes is often as little as the amount in one chromosome (Ege and Ringertz, 1974). However, a more involved procedure is used to prepare microcells.

The first step in microcell production is the induction of micronucleation. In rodent cells, this procedure is usually carried out using mitotic inhibitors such as colchicine (Ege *et al.*, 1977). Other agents which have caused micronucleation are X-irradiation, Colcemid, griseofulvin, and vinblastine sulfate. Although the steps in micronucleation are not understood completely, it appears that the mitotic inhibitors cause disruption of the microtubules of the cell, thus causing the condensed metaphase chromosomes to remain scattered throughout the cell. The isolated chromosomes or groups of chromosomes act as foci for nuclear membrane formation, resulting in many small amounts of chromatin enclosed in nuclear membrane spread throughout the cell (these are known as microcells). The size of each microcell (depending on the cell and the treatment) can vary from minicell size (the entire chromatin) to microcells the size of an individual chromosome. Microcells are then collected by centrifugation in cytochalasin B medium exactly as are minicells. These microcells behave exactly as minicells and can be used for hybridization (Fig. 10B). Microcells, once collected, can be roughly classified and separated according to size (and amount of chromatin) by sedimentation at unit gravity through a linear gradient of 1–3% bovine serum albumin (Hecht *et al.*, 1975; Fornier and Ruddle, 1977a; Sundar-Raj *et al.*, 1977). This procedure enables the researcher to fuse whole cells with different size classes of microcells and, to an extent, to control the number of chromosomes added to the hybridization event.

Human microcell preparation must be carried out using different procedures because most mitotic inhibitors have an adverse effect on human cells. Schor *et al.* (1975) treated human cells (HeLa cells) with nitrous oxide under pressure (5 atm.) followed by a 9-hour treatment at 4°C to obtain microcells. This procedure causes micronuclei to "bud"

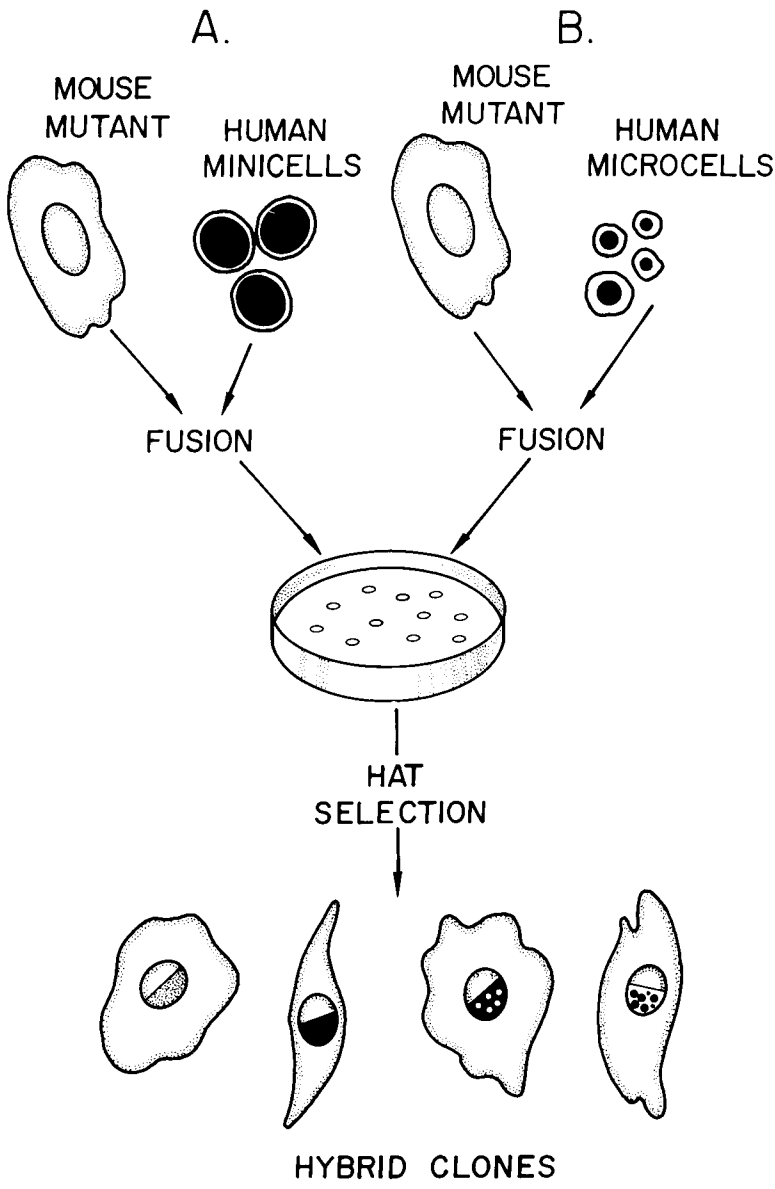

FIG. 10. Minicell and microcell mediated somatic cell hybridization. Human minicells (A) and microcells (B) are fused much the same way as with whole cells (Fig. 4). Selection will be against only the mouse parent since the human cell fragments are not viable after about 24–48 h.

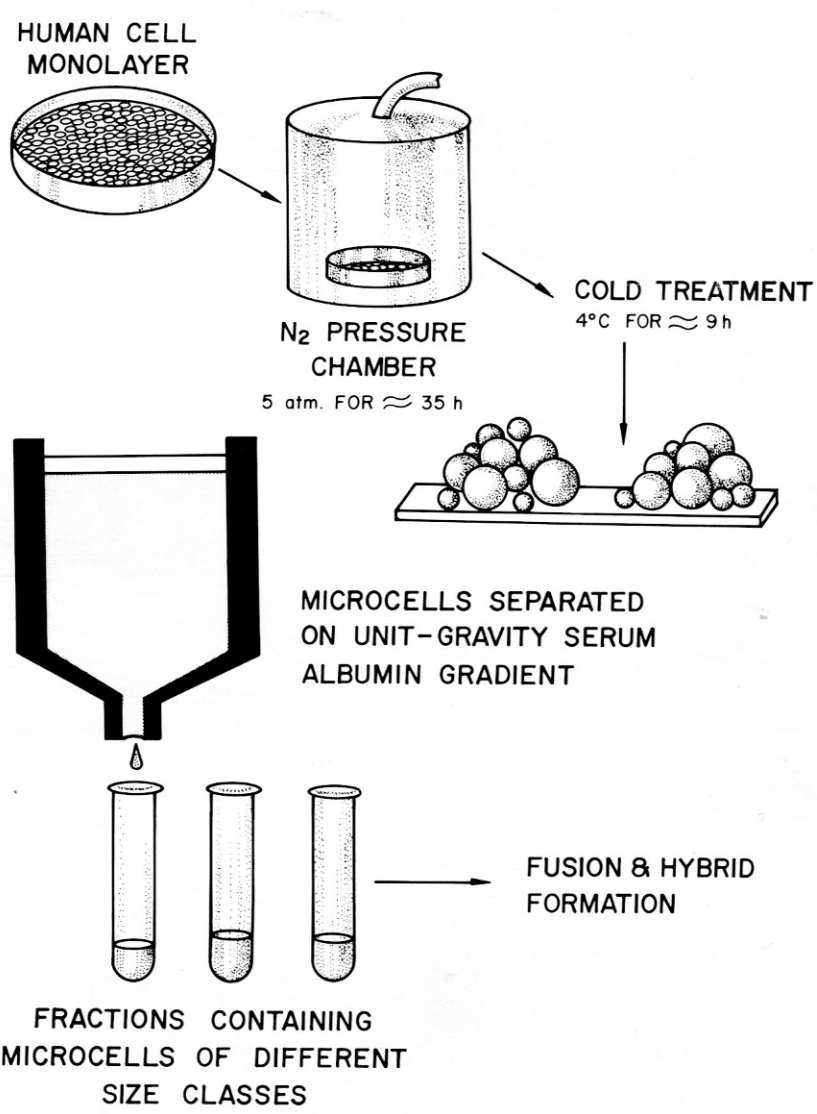

FIG. 11. Preparation of human microcells. Incubation of the cells in nitrous oxide under 5 atmospheres of pressure followed by a cold shock treatment causes "budding" of micronuclei and subsequent shedding of these microcells into the medium. Microcells are size-distributed through a serum albumin gradient and used for hybridization (Fig. 10B).

from the cell (Johnson *et al.*, 1975) so they can be easily detached and collected (Fig. 11). This procedure has been used to isolate a hybrid containing only human chromosome 17 in a mouse LM(TK$^-$)–human hybrid (Sundar-Raj *et al.*, 1977).

The advantage of microcell hybridization lies in the speed with which stable hybrids can be produced. If very few chromosomes are incorporated into a hybrid, stabilization of the hybrid genotype occurs much faster than would be observed if all 46 human chromosomes were present.

3. Isolated Metaphase Chromosome-Mediated Gene Transfer Hybrids

Recently, a large number of publications have appeared demonstrating the transfer of genetic information into recipient cells via isolated metaphase chromosomes (McBride and Ozer, 1973; Wullems *et al.*, 1975, 1976a,b, 1977; Willecke and Ruddle, 1975; Burch and McBride, 1975; Spandidos and Siminovitch, 1977a,b; Willecke *et al.*, 1976; Fournier and Ruddle, 1977b). In almost every instance, the gene transferred was a selectable marker, i.e., TK or HGPRT. Apparently, only a small piece of chromosome is transferred. This piece amounts to less than 1% of the human genome (Willecke and Ruddle, 1975) and has been termed the transgenome. Cotransfer of more than one gene has been demonstrated [TK and galactokinase (GALK); Willecke *et al.*, 1976; Burch and McBride, 1975; Ruddle and McBride, 1976], but transfer of intact, stable chromosomes appears to be a very rare occurrence (Wullems *et al.*, 1976b).

Most methods for isolating metaphase chromosomes follow that of Maio and Schildkraut (1967) or modifications of this method. Briefly, this procedure involves an initial administration of mitotic block to cultured cells to build up a large number of cells in metaphase, followed by gentle lysis of the cells and centrifugal purification of the metaphase chromosomes. These metaphase chromosomes are then suspended in culture medium and mixed with freshly trypsin-treated recipient cells and poly-L-ornithine (poly-L-ornithine stimulates phagocytosis by the recipient cells). The chromosome–cell mixture is then incubated for a selected time with mild shaking, after which the cells are plated on tissue culture dishes; hybrids are selected in HAT medium (or any other selection medium). Colonies are observed at very low hybridization frequencies (about 10^{-6} to 10^{-7}) and must be grown in selective medium for the first few months after hybridization to allow stabilization of the transgenome (Fig. 12).

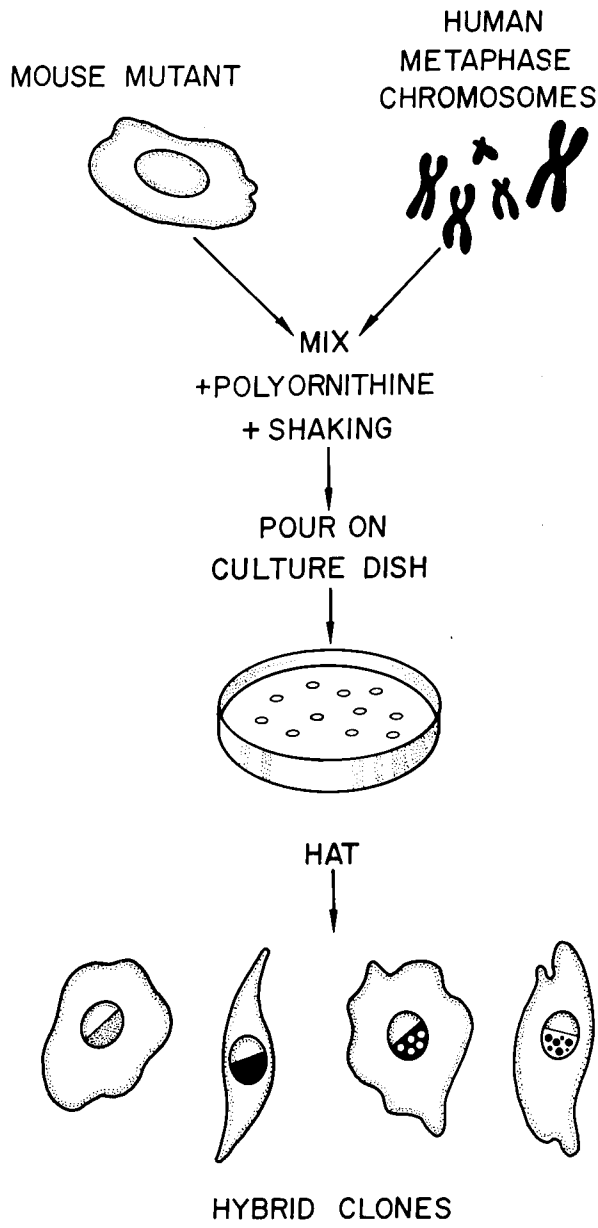

FIG. 12. Isolated metaphase-chromosome-mediated "hybrid" production. Isolated human metaphase chromosomes (prepared as described in the text) are mixed in suspension with mouse cells and polyornithine (to induce chromosome phagocytosis). The cells are incubated with chromosomes and then plated and hybrids selected in HAT medium (or other selection medium).

This procedure is valuable because very small amounts of "foreign" genetic information can be incorporated into recipient cells. Coexpression of tightly linked loci can be examined using metaphase chromosome gene transfer (Willecke *et al.,* 1976), and possibly, isolation and purification of the transgenome can be achieved if the foreign DNA is sufficiently different from the recipient's DNA.

Trispecific microcell hybrids have been produced from a human–mouse gene-transfer cell line into a Chinese hamster recipient line (Fournier and Ruddle, 1977a). This line was produced by initially isolating a mouse cell line that expressed the human form of HGPRT (selected through a gene transfer step involving mouse $HGPRT^-$ recipient and isolated human chromosome). The mouse line was then microcell hybridized with a Chinese hamster $HGPRT^-$ cell line, resulting in a stable Chinese hamster–mouse hybrid expressing human HGPRT. Since the incorporation of the transgenome is random for recipient cells, it is theoretically possible to use this procedure to establish a panel of mouse–human hybrids which consists of 24 clones, each of which contains only 1 specific human chromosome. For instance, if skin fibroblasts are grown from a human Lesch–Nyhan syndrome source (which is genetically deficient in HGPRT), these cells would serve as recipients for a Chinese hamster metaphase chromosome gene transfer. A large number of human cells expressing Chinese hamster HGPRT (selected with HAT medium) is then isolated and used for microcell hybridization experiments using mouse $HGPRT^-$ cells as the intact parent. Again, large numbers of hybrid clones are picked and maintained as hybrids (with HAT medium), and each clone is assayed for the presence of a particular human chromosome. Since the Chinese hamster HGPRT transgenome is randomly incorporated into human chromosomes in the initial gene-transfer experiment, it is possible to select microcell hybrids which contain only one human chromosome, different for each clone picked. With this panel of hybrids, gene assignment becomes a simple matter of testing each line for the products of the gene in question and assigning the gene to the individual human chromosome present.

IV. Somatic Cell Genetics of Connective Tissue Proteins

Utilizing the genetic methods outlined in this chapter, we have spent the past few years investigating the chromosomal localization of the genes for connective tissue proteins, specifically the collagens. The collagens are the most abundant and widespread connective tissue protein in the body. The data and thoughts presented in this section con-

stitute only the beginning of our work and indicate directions of study rather than completed work.

A. Gene Assignment of Type I Procollagen–Collagen

Type I collagen is produced by many different tissues in the body (Hay *et al.,* 1979), and the structure of these type I collagens appears to be identical for each tissue (at the level of CNBr peptide analysis). However, the extreme diversity of tissues containing type I collagen (i.e., from bone to cornea) suggests that a family of type I collagens exists, having different genetic origins. One way of testing this hypothesis is by carrying out gene assignment studies for each type I collagen in each tissue. For example, if the gene for type I collagen in skin is on a different chromosome than the gene for type I collagen in bone, the two collagens are genetically distinct and may be under completely different regulatory controls.

1. Skin Type I Procollagen Gene Assignment

Our initial studies were carried out with mouse–human skin fibroblast hybrids or mouse–human fetal lung fibroblast hybrids. Hybridization was carried out using the classic procedure outlined in Fig. 4. Once hybrid clones were obtained, chromosome analysis was carried out using isozyme analysis of 23 separate enzymes for 18 different human chromosomes. Karyology was carried out on metaphase chromosome spreads using quinacrine and alkaline Giemsa (G-11) differential staining procedures (Friend *et al.,* 1976a,b).

Type I procollagen was assayed using a specific antibody to purified human type I procollagen (Church and Tanzer, 1975) prepared in rabbits (Park *et al.,* 1975). Since both mouse and human type I procollagen will probably be produced in some hybrids (Fig. 13), procollgen antibody was tested against purified mouse type I procollagen and against concentrated culture medium from mouse A9 ($HGPRT^-$) and LM(TK^-) cells (which were used as the mouse parent in the hybrids). Figure 14 shows that our anti-human type I procollagen gave a strong reaction to purified human type I procollagen but no reaction was observed when the anti-procollagen was tested against mouse procollagen samples (Fig. 14a). The hybrids were tested by collecting serum-free medium from each hybrid and concentrating the medium to a small volume, or the procollagen was purified from the medium samples before testing (Church *et al.,* 1974). Ouchterlony double immunodiffusion was used to

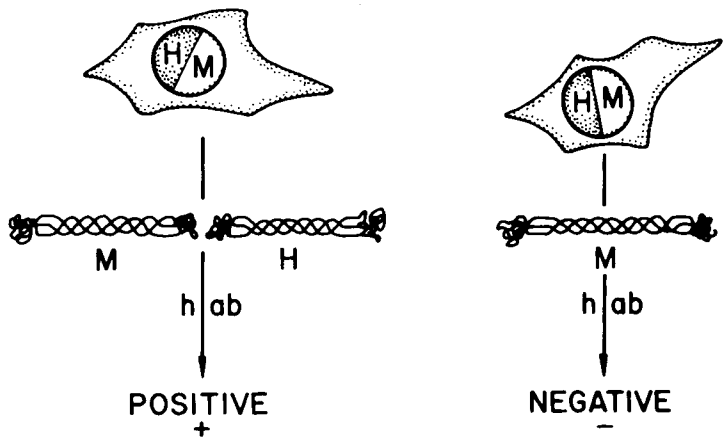

FIG. 13. Schematic representation of two hybrid lines, one producing mouse and human procollagen, and one producing only mouse procollagen. The specific antibody to human procollagen will detect those hybrids which produce human procollagen. Mouse procollagen does not react with our human procollagen antibody.

test each hybrid sample. Positive or negative scores were determined by the presence or absence of precipitation band 5 observed in the gel (Fig. 14b). Table IV presents the correlation of human chromosome present in each hybrid clone with human skin type I procollagen production. In this table concordant clones (those expressing skin procollagen production *and* the particular chromosome, or those clones negative for both chromosome and procollagen) are compared with discordant clones (positive for procollagen and negative for chromosome, or vice versa). As can be seen, only human chromosome 17 expressed complete concordancy. This result strongly suggested that human chromosome 17 contains the gene coding for human skin type I procollagen (Sundar-Raj *et al.*, 1976, 1977). A microcell-mediated hybrid between mouse cells and human microcells was prepared which contained only human chromosome 17. This hybrid was also positive for human skin type I procollagen, confirming the assignment of the human skin type I procollagen gene to human chromosome 17 (Sundar-Raj *et al.*, 1977). Another hybrid cell line, which contained only the long arm of human chromosome 17 translocated onto a mouse chromosome (Fig. 15; McDougall, *et al.*, 1973; Elsevier *et al.*, 1974), was then tested for type I procollagen production. Again positive results for the production of human type I procollagen were obtained. With all of the

TABLE IV

CORRELATION OF HUMAN SKIN TYPE I PROCOLLAGEN SYNTHESIS WITH THE PRESENCE OF INDIVIDUAL HUMAN CHROMOSOMES

Human chromosome	Human skin type I procollagen/chromosome[a]				Concordant clones (+/+ and −/−)	Discordant clones (+/− and −/+)
	+/+	+/−	−/+	−/−		
1	0	4	1	7	7	5
2	2	2	2	6	8	4
3	0	4	3	5	5	7
4	0	4	4	4	4	8
5	2	2	2	6	8	4
6	1	3	0	8	9	3
7	2	2	2	6	8	4
8	0	4	0	8	8	4
9	0	4	0	8	8	4
10	0	4	2	6	6	6
11	2	2	0	8	10	2
12	2	2	3	5	7	5
13	2	2	0	8	10	2
14	1	3	4	4	5	7
15	1	3	1	7	8	4
16	1	3	2	6	7	5
17	4	0	0	8	12	0
18	2	2	1	7	9	3
19	2	2	2	6	8	4
20	2	2	2	6	8	4
21	2	2	5	3	5	7
22	0	4	1	7	7	5
X	1	3	4	4	5	7

[a] +/+, Human skin type I procollagen positive/chromosome positive; +/−, human skin type I procollagen positive/chromosome negative; −/+, human skin type I procollagen negative/chromosome positive; −/−, human skin type I procollagen negative/chromosome negative.

hybrid clones that were positive for both human chromosome 17 and human type I procollagen production, we were able to back select cells that have lost human chromosome 17 by growing the hybrid clones in medium containing BUdR. All of these clones lost the ability to produce human type I procollagen concomitant with the loss of human chromosome 17.

Since the human skin type I procollagen gene appeared to be localized on human chromosome 17, we investigated other functions previously assigned to this chromosome. As seen in Table III, TK and

GALK have previously been mapped to the long arm of chromosome 17 (Fig. 16). Also, the adenovirus 12 modification site was assigned to a small area in the long arm of human chromosome 17 (McDougall, 1971). Treatment of cultured cells with adenovirus 12 causes the long

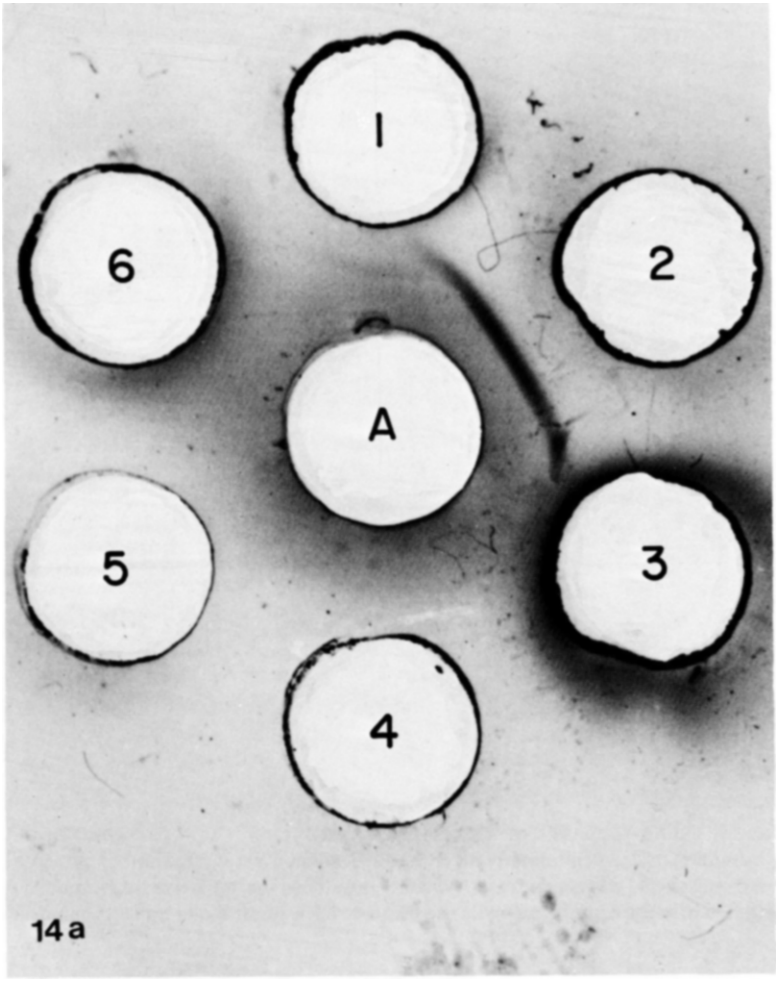

FIG. 14. Ouchterlony double immunodiffusion identification of human procollagen. Human procollagen was purified and antibody produced using methods cited in the text. (a) Reaction of purified human skin type I procollagen (well 2) with antibody to this procollagen, produced in rabbits (center well). Concentrated medium from mouse A9 (well 3) and mouse LM (well 4) cells did not show cross-reaction as well as purified mouse type I procollagen (well 5) and mouse procollagen purified from the medium of mouse A9 cells (well 6). See p. 134 for Fig. 14b.

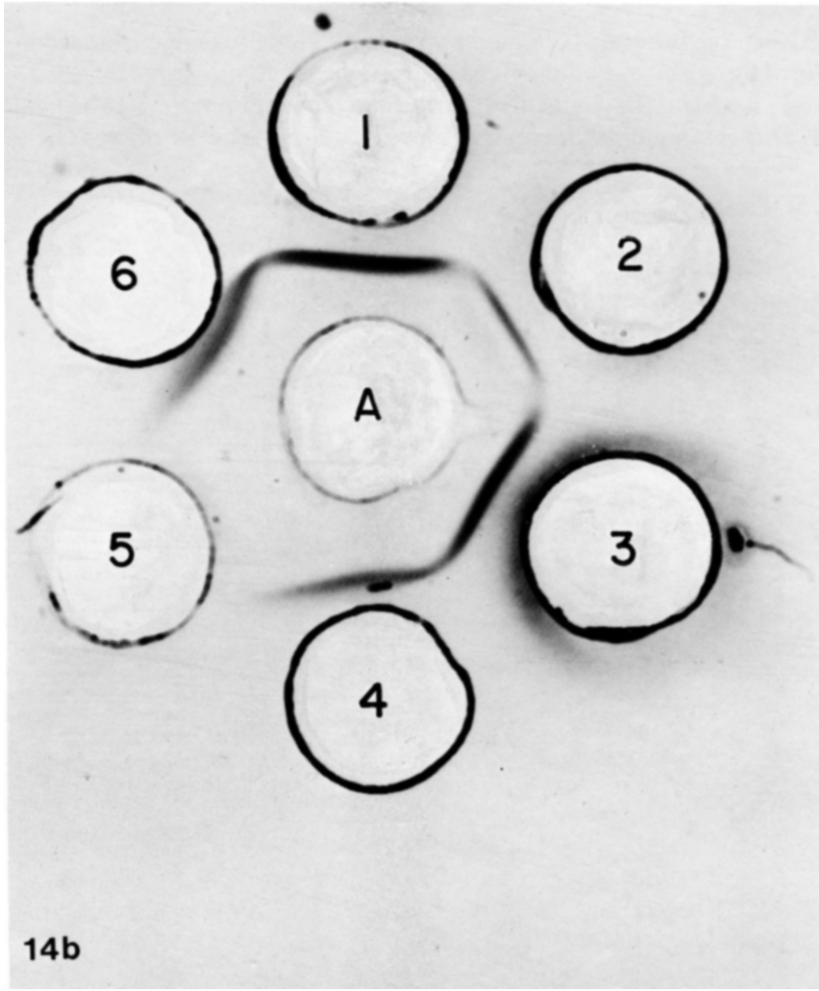

FIG. 14. (b) Anti-human type I procollagen in the center well was reacted against purified human type I procollagen (well 1) and the concentrated medium or purified procollagen from several mouse–human hybrid clones. Well 5 is negative for human type I procollagen, while the remaining wells are positive for human type I procollagen production.

arm of human chromosome 17 to open up or uncoil (Fig. 17), followed by a fragmentation of the chromosome. This virus treatment was utilized with the hybrid containing the long arm of human chromosome 17 translocated to a mouse chromosome in order to prepare a series of hybrid clones that contained different length pieces of the long arm of human chromosome 17 (Fig. 18). These clones were previously used to

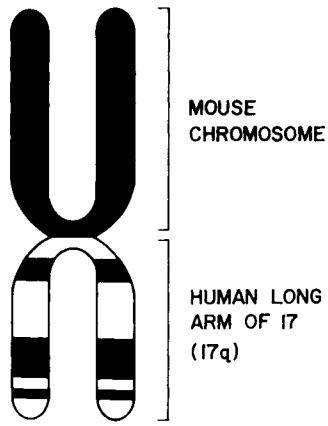

FIG. 15. Scheme of translocated portion of human chromosome 17 (long arm) onto a mouse chromosome. The human centromere is attached to the mouse terminal end with the entire long arm of 17 (banded area) translocated.

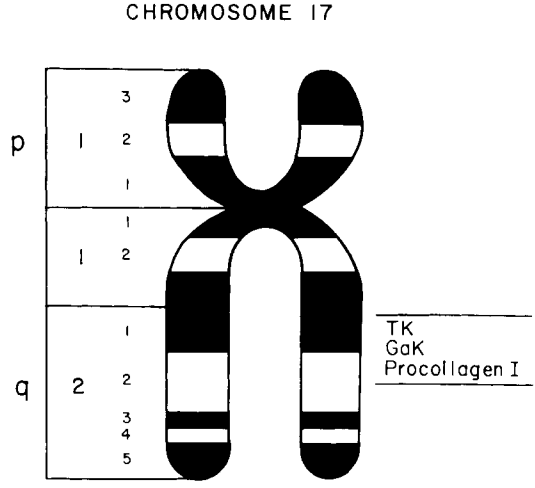

FIG. 16. Schematic representation of human chromosome 17 with the "typical" banding pattern found from Q- or G-banding stains. Thymidine kinase (TK) and galactose kinase (GaK; in text, GALK) have been assigned to bands q21-q22 in the long arm of chromosome 17.

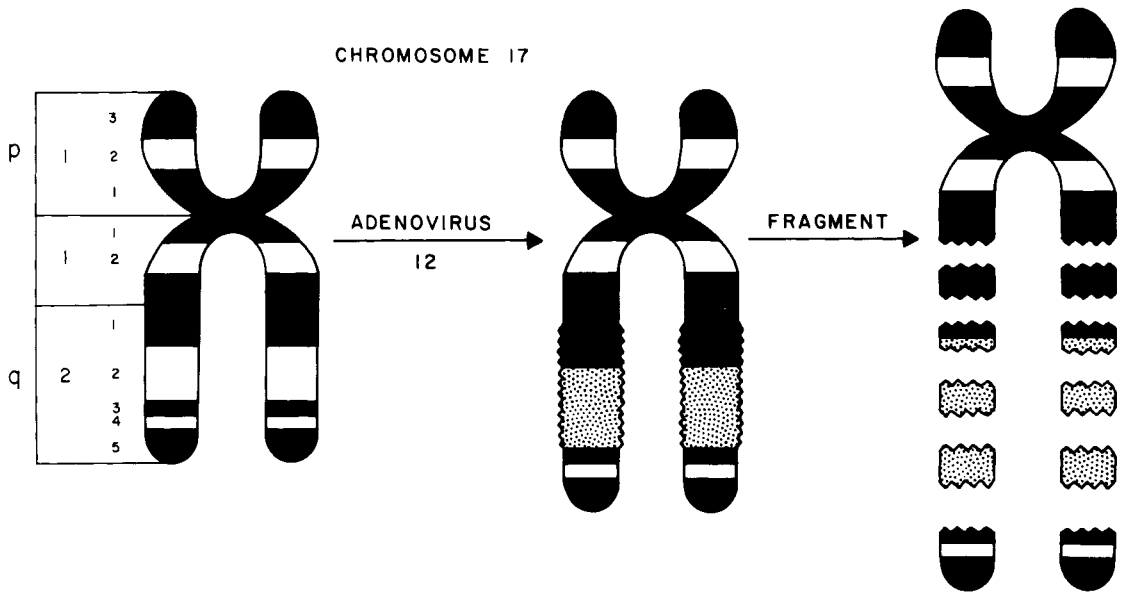

FIG. 17. Human chromosome 17 before and after treatment with adenovirus 12. Initial virus treatment causes an "uncoiling" of a small section in the long arm of chromosome 17, leading to multiple chromosome breaks.

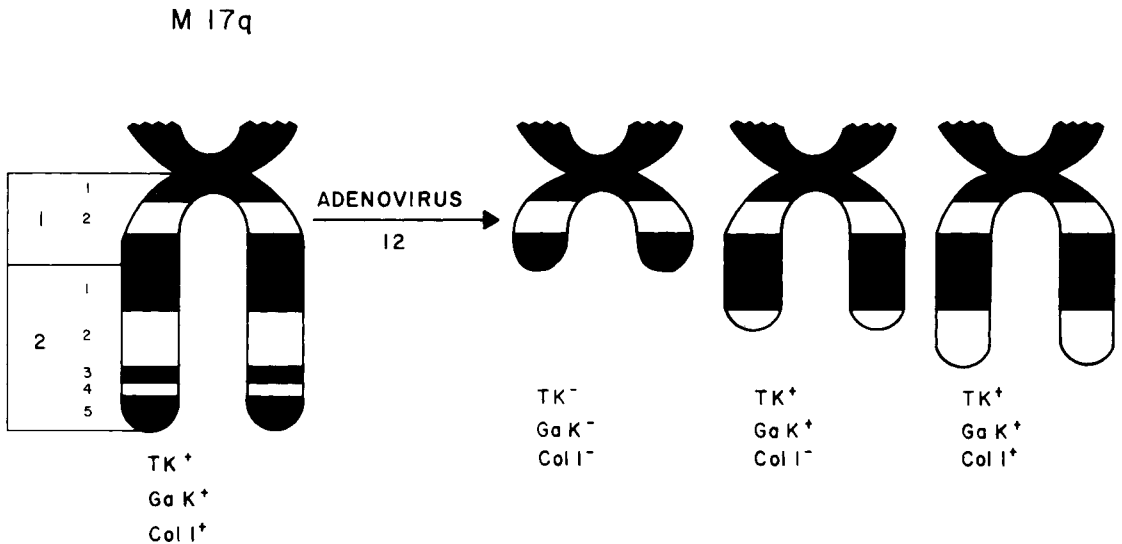

FIG. 18. Schematic representation of clones isolated from adenovirus 12 treatment of the mouse–human hybrid line having the long arm of chromosome 17 translocated to a mouse chromosome (Fig. 15). Clones were picked having different arm lengths of chromosome 17 left. Assays for TK, GaK (in text, GALK), and procollagen I were made on each clone.

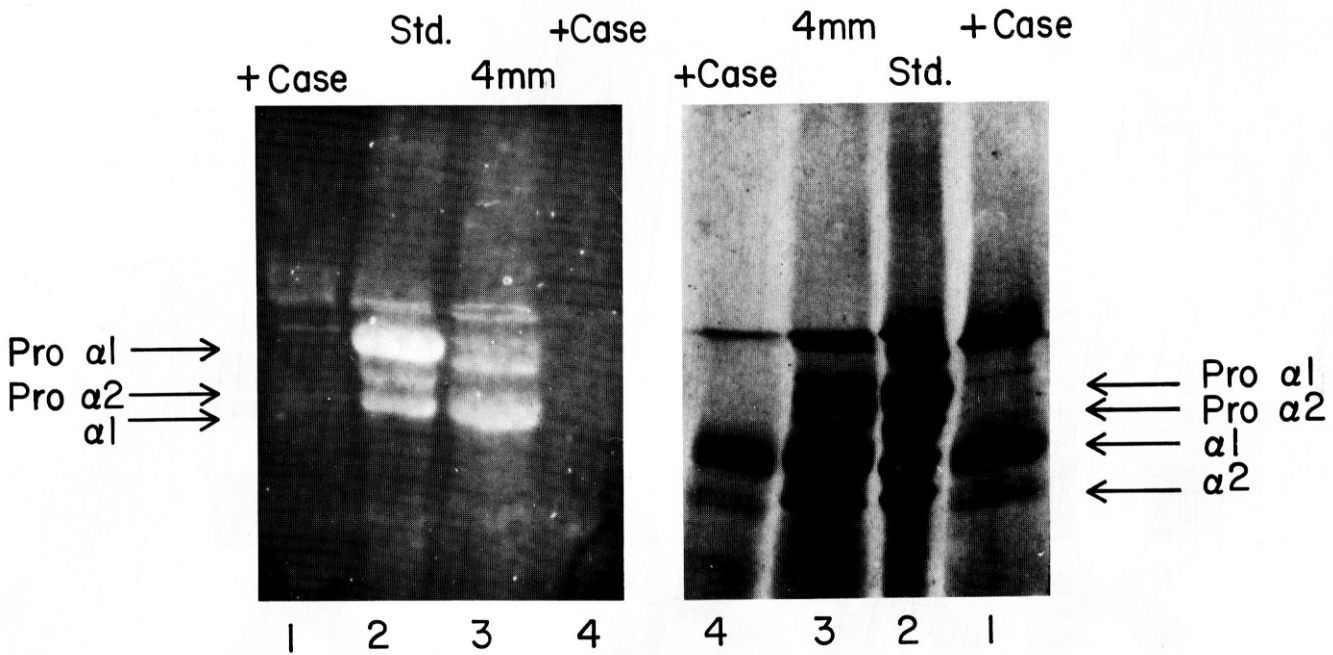

FIG. 19. Fluorescent visualization of human type I procollagen and collagen chains (left panel) separated on SDS–polyacrylamide slab gels. Human-specific type I procollagen antibody was used to immunostain the separated pro-α and α chains. Right panel depicts an identical gel stained with Coomassie blue. Pro-α1, pro-α2, α1, and α2 chains are identified on the gels. Stained bands migrating near α1 and α2 chains in the collagenase-treated gels (+Case) are collagenase subunits. No immunostained bands are observed in the pro-α or α chain regions in collagenase-treated gels (left panel). Purified human type I procollagen was run as standard (Std.). Procollagen from clone 4mm-100 was used as test material.

localize the genes for TK and GALK to a very small region in the long arm of human chromosome 17 (Fig. 16). We tested these clones for the production of the previously cited isozymes and human skin type I procollagen. The results, shown in Fig. 16, confirm the localization of the gene for human skin type I procollagen in the same region as TK and GALK.

One final experiment involving gene assignment for human skin type I procollagen involved the preparation of isolated human metaphase chromosome gene transfer hybrids in a mouse recipient. Mouse LM(TK$^-$) cells were used as the recipient since they selectively retain the TK gene in the hybrid when HAT medium is used. It had been shown earlier (Burch and McBride, 1975; Ruddle and McBride, 1977; Willecke *et al.*, 1976) that TK and GALK were tightly linked and could often be cotransferred in chromosome-mediated gene transfer experiments. We have investigated the frequency of cotransfer of human type I procollagen with TK and GALK and have found that cotransfer does take place, and the stable clones obtained maintain the genes for human GALK, TK, and procollagen type I (Klobutcher and Ruddle, 1979).

We were able to demonstrate that both human pro-α1 and pro-α2 chains were produced in these hybrids by first separating the chains on SDS–polyacrylamide slab gels, followed by immunostaining the separated chains with fluorescent-labeled anti-human procollagen (Fig. 19; Church *et al.*, 1980).

2. Ocular Type I Procollagen Gene Assignment

Human corneal stroma, conjunctival, and scleral fibroblasts were hybridized with mouse A9 (HGPRT$^-$) and LM(TK$^-$) cell lines and hybrid clones were isolated. Our preliminary data have resulted in a chromosomal assignment for the conjunctival and scleral type I procollagen gene to human chromosome 17 (apparently the same gene as for skin type I procollagen). In the conjunctival–mouse hybrid we were able to isolate 4 clones which contain human chromosome 17 as the only human contribution. Each of these clones was positive for human type I procollagen production. Several clones of scleral–mouse hybrids which contained the long arm of human chromosome 17 and no other human chromosome material were isolated (Fig. 20). These clones were also positive for human type I procollagen. We are now in the process of adenovirus-12-treating these translocation hybrids in an effort to confirm the regional assignment for scleral type I procollagen.

Our results using human corneal stroma fibroblast–mouse hybrids

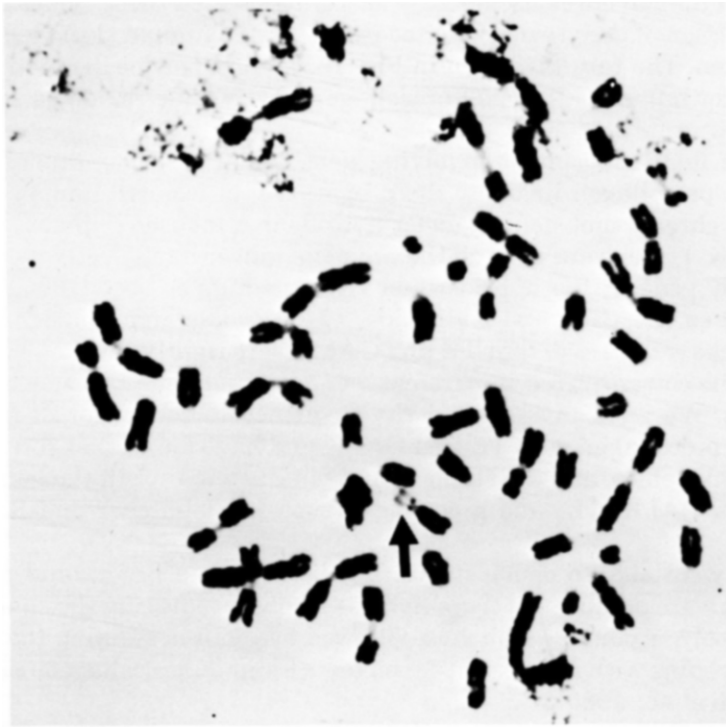

FIG. 20. Giemsa-11 staining of a human scleral fibroblast–mouse hybrid demonstrating a translocation of the long arm of human chromosome 17, including the centromere, to a mouse chromosome (arrow).

have indicated that the gene for corneal type I procollagen *does not* map to human chromosome 17. Thus, there appears to be a different genetic origin for corneal type I procollagen than for skin, lung, conjunctival, and scleral type I procollagen (Church and Rohrbach, 1978a,b; Church *et al.*, 1980). Table V shows a concordancy plot of corneal type I procollagen production versus human chromosome present in the hybrids. Human chromosome 7 demonstrates the highest frequency of concordant (+/+) clones. The two disparate clones are probably due to rearrangements or translocations within this particular hybrid. Thus, a tentative assignment for the gene coding for corneal type I procollagen can be made to human chromosome 7 (Fig. 21). This difference in gene assignment for corneal type I procollagen and other tis-

sue type I procollagen genes indicates the possibility of a family of type I collagens, each one of these being tissue specific, and may be related to the functional differences between the collagen fibers of corneal stroma and skin.

Another chromosome assignment for human skin type I collagen has been proposed by Sykes and Solomon (Sykes and Solomon, 1978; Solomon and Sykes, 1979). Using anti-human type I collagen prepared in rabbits, they determined that the human type I collagen structural

TABLE V

CORRELATION OF HUMAN CORNEAL STROMA TYPE I PROCOLLAGEN SYNTHESIS WITH THE PRESENCE OF INDIVIDUAL HUMAN CHROMOSOMES

Human chromosome	Human corneal stroma type I procollagen/chromosome[a]				Concordant clones (+/+ and −/−)	Discordant clones (+/− and −/+)
	+/+	+/−	−/+	−/−		
1	2	3	1	19	21 (19−/−)	4
2	1	4	1	19	20 (19−/−)	5
3	2	3	4	16	18 (19−/−)	7
4	2	4	2	18	19 (19−/−)	6
5	1	4	5	15	16 (15−/−)	9
6	1	4	2	18	19 (18−/−)	6
7	3	0	2	20	23 (20−/−)	2
8	3	2	4	16	19 (16−/−)	6
9	1	4	3	17	18 (17−/−)	7
10	2	3	3	17	19 (17−/−)	6
11	0	5	2	18	18 (18−/−)	7
12	3	2	7	13	16 (13−/−)	9
13	1	4	7	13	14 (13−/−)	11
14	4	1	15	5	9	16
15	2	3	7	13	15 (13−/−)	10
16	0	5	2	18	18 (18−/−)	7
17	0	5	2	18	18 (18−/−)	7
18	2	3	6	14	16 (14−/−)	9
19	2	3	10	10	12	13
20	4	1	13	7	11	14
21	4	1	10	10	14	11
22	1	4	3	17	18	7
X	5	0	11	9	14	11

[a] +/+, Human corneal stoma type I procollagen positive/chromosome positive; +/−, human corneal stroma type I procollagen positive/chromosome negative; −/+, human corneal stroma type I procollagen negative/chromosome positive; −/−, human corneal stroma type I procollagen negative/chromosome negative.

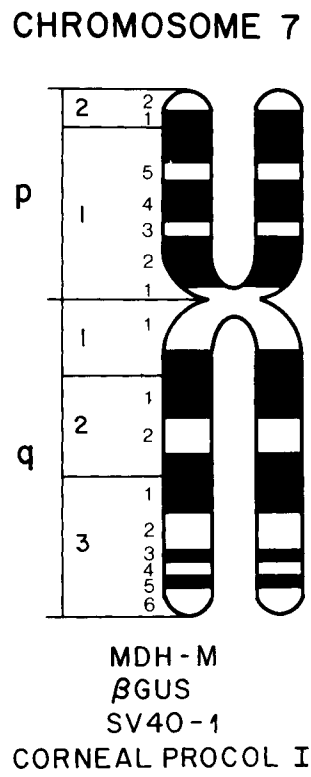

FIG. 21. Schematic representation of human chromosome 7, listing genes mapped to this chromosome.

gene was on human chromosome 7 (Sykes and Solomon, 1978). Further, they presented evidence that both the type I $\alpha 1$ and $\alpha 2$ chain genes map to chromosome 7 (Solomon and Sykes, 1979). This apparent inconsistency with results obtained in our laboratory has not been satisfactorily explained, unless one attributes it to multiple genes on different chromosomes for the collagens.

B. Human Type III Procollagen Gene Assignment

We have recently assayed a large panel of mouse–human fibroblast hybrids for the production of human type III procollagen, essentially as was carried out for type I procollagen mapping. We have used fibroblasts from various human cell sources: adult (30-year-old female skin), young male skin (2½ years and 10 months of age), fetal tissue (lung, skin), and amniotic fluid. Our results, still tentative, indicate

that hybrids produced from young or adult human cells yield a gene assignment for human type III procollagen to human chromosome 18, while fetal human cell hybrids map human type III procollagen to human chromosome 4. This observation indicates a "switching" of procollagen genes during development and may be quite important in terms of the development of certain tissues.

Solomon and Sykes (1979) used anti-human type III collagen antibodies combined with interrupted gel electrophoresis (Sykes *et al.*, 1976) to assign tentatively the human type III collagen gene to human chromosome 7. Again, a discrepancy exists between laboratories regarding the gene assignment for human type III procollagen–collagen.

C. *Human Basement Membrane Collagen (Type IV) Gene Mapping*

Kefalides (1979) reported a preliminary identification of the gene for human basement membrane collagen (type IV) on human chromosome 17. This identification was based on immunofluorescent staining of human endothelial cell–mouse hybrids with anti-basement membrane collagen. A strong correlation between positively immunostained hybrid cells and the presence of human chromosome 17 was observed. Also, back selection of these hybrids in BUdR-containing medium caused the hybrids to stop reacting with the basement membrane collagen antibody. It was suggested that the gene for human basement membrane collagen or a gene controlling basement membrane collagen synthesis may be located on human chromosome 17.

D. *Human Fibronectin Gene Mapping*

Fibronectin (FN), also called large external transformation sensitive (LETS) protein, cell surface protein (CSP), fibroblast surface antigen, and cell adhesion factor, is a large glycoprotein that constitutes an important part of the surface of many cell types (Yamada and Olden, 1978). Fibronectin has been shown to mediate the attachment of fibroblasts to collagen substrates (Klebe, 1974) and appears to be reduced in amount on the surface of transformed cells (Vaheri and Ruoslahti, 1975; Hynes, 1976).

Preliminary localization of the fibronectin gene on human chromosome 8 has been reported by Owerback *et al.* (1978), who used cell surface labeling with peroxidase to detect bound fibronectin. The murine parental cell line used in their study lacked cell surface fibronectin, whereas certain of the hybrids studied had the protein on their surfaces. Since transformed mammalian cells sometimes produce but do

not retain fibronectin (Vaheri and Ruoslahti, 1975), it is possible that a receptor for fibronectin, rather than fibronectin itself might have been assayed. For this reason we have carried out related studies using immunoassays to quantitate murine and human fibronectin separately. Our results confirmed Owerback's assignment of the location of the human fibronectin gene to chromosome 8 (Rennard *et al.*, 1980).

Using species-specific antibodies to both human and mouse fibronectin, we have quantitatively assayed both human and mouse fibronectin in 32 separate hybrid clones obtained from human skin fibroblasts, human corneal stroma fibroblasts, and human scleral fibroblasts hybridized with mouse A9 ($HGPRT^-$), LM(TK^-), and B82 (TK^-) cell lines. All hybrids secreted mouse fibronectin into the culture medium at a rate of about 0.5–3.0 μg/ml/48 h. Human positive fibronectin hybrids produced 0.04–0.21 μg/ml/48 h of human fibronectin. Assays of human chromosome complement and human fibronectin production in the hybrid lines (Table VI) indicate complete concordancy with human fibronectin production and the presence of human chromosome 8 (Rennard *et al.*, 1980). Other laboratories (Eun and Klinger, 1979, 1980; Smith *et al.*, 1979) have demonstrated synteny between human fibronectin production and human chromosomes 3 and 11 in human–mouse somatic cell hybrids. Both groups assayed for the presence of human fibronectin by using immunofluorescent localization on cultured cells. As mentioned earlier, these types of assays may measure a cell receptor for fibronectin rather than the production of fibronectin itself and may thus be deomonstrating a controlling or modifying factor instead of the structural gene. Alternatively, there may be several different cell surface attachment proteins having different chromosomal localizations.

V. Conclusions

This relatively brief outline covering uses of and techniques involved in somatic cell hybridization was not intended to be an all-encompassing description of every aspect of hybridization. It was intended to introduce this area of research to connective tissue biologists and to act as a reference source for those who wish to deal with this topic in greater detail. The use of somatic cell hybridization in the connective tissue field is still in its infancy, with many very exciting areas open to the connective tissue biologist. Obviously, the small amount of data presented here only demonstrates the uses of this technique. It is hoped that the information contained herein will stimulate others in the connective tissue field to make use of these very powerful proce-

TABLE VI

CORRELATION OF THE PRODUCTION OF HUMAN FIBRONECTIN WITH INDIVIDUAL HUMAN CHROMOSOMES IN HUMAN–MOUSE HYBRIDS

Human chromosome	Human fibronectin/chromosome[a]				Concordant clones (+/+ and −/−)	Discordant clones (+/− and −/+)
	+/+	+/−	−/+	−/−		
1	2	5	2	23	25	7
2	3	4	1	24	27	5
3	1	6	4	21	22	10
4	6	1	4	21	27	5
5	1	6	5	20	21	11
6	1	6	5	20	21	11
7	4	3	7	18	22	10
8	7	0	0	25	32	0
9	1	6	1	24	25	7
10	1	6	7	18	19	13
11	0	7	5	20	20	12
12	3	4	6	19	22	10
13	0	7	3	22	22	10
14	5	2	11	14	19	13
15	2	5	7	18	20	12
16	0	7	4	21	21	11
17	0	7	9	16	16	16
18	2	5	5	20	22	10
19	3	4	9	16	19	13
20	4	3	7	18	22	10
21	4	3	7	18	22	10
22	1	6	2	23	27	5
X	6	1	16	9	15	17

[a] +/−, Human fibronectin positive/chromosome positive; +/−, human fibronectin positive/chromosome negative; −/+, human fibronectin negative/chromosome positive; −/+, human fibronectin negative/chromosome negative.

dures in order to better understand the numerous and intricate workings of all connective tissues.

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References

Aden, D. P., and Knowles, B. B. (1976). *Immunogenetics* **3,** 209–221.

Ahkong, Q. F., Cramp, F. C., Fisher, D., Howell, J. I., and Lucy, J. A. (1972). *J. Cell Sci.* **10,** 769–787.

Akers, T. G., and Cunningham, C. H. (1968). *Arch. Gesamte Virusforsch.* **25,** 30–37.

Appleyard, G., Westwood, J. C., N., and Zwartouw, H. T. (1962). *Virology* **18,** 159–169.

Arrighi, F., and Hsu, T. C. (1971). *Cytogenetics* **10,** 81–86.

Baker, R. M., Brunette, D. M., Mankovitz, R., Thompson, L. H., Whitmore, G. F., Siminovitch, L., and Till, J. E. (1974). *Cell* **1,** 9–21.

Barski, G., Sorieul, S., and Cornefert, F. (1960). *C. R. Hebd. Seances Acad. Sci.* **251,** 1825–1827.

Beaudet, A., Roufa, D., and Caskey, C. (1973). *Proc. Natl. Acad. Sci. U.S.A.* **70,** 320–324.

Bennet, L. L., Schnebli, H. P., Vail, M. H., Allan, P. W., and Montgomery, J. H. (1966). *Mol. Pharmacol.* **2,** 432–443.

Blough, H. A. (1964). *Cell. Biol. Myxovirus Infect. Ciba Found. Symp.,* 120–143.

Bobrow, M., and Cross, J. (1974). *Nature (London)* **251,** 77–79.

Bobrow, M., Modan, K., and Pearson, P. L. (1972). *Nature (London) New Biol.* **238,** 122–124.

Boone, C. M., Chen, T.-R., and Ruddle, F. H. (1972). *Proc. Natl. Acad. Scci. U.S.A.* **69,** 510–514.

Bridges, B., Huckle, J., and Ashwood-Smith, M. (1970). *Nature (London)* **226,** 184–186.

Burch, J. W., and McBride, O. W. (1975). *Proc. Natl. Acad. Sci. U.S.A.* **72,** 1797–1801.

Carlsson, S.-A., Luger, O., Ringertz, N. R., and Savage, R. E. (1974). *Exp. Cell Res.* **84,** 47–55.

Carter, S. B. (1967). *Nature (London)* **213,** 261–266.

Caspersson, T., Farber, S., Foley, G. E., Kudynowski, J., Modest, E. J., Simonsson, E., Wagh, U., and Zech, L. (1968). *Exp. Cell Res.* **49,** 219–222.

Caspersson, T., Zech, L., Johannson, C., and Modest, E. J. (1970). *Chromosoma* **30,** 215–227.

Caspersson, T., Zech, L., Harris, H., Wiener, F., and Klein, G. (1971). *Exp. Cell. Res.* **65,** 475–478.

Chan, T.-S., and Creagan, R. P. (1976). *Cytogenet. Cell Genet.* **16,** 378–381.

Chu, E., and Malling, H. (1968). *Proc. Natl. Acad. Sci. U.S.A.* **61,** 1306–1312.

Church, R. L., and Rohrbach, D. H. (1978a). *Invest. Ophthalmol. Visual Sci. Suppl.* **17,** 211.

Church, R. L., and Rohrbach, D. H. (1978b). Third International Congress of Eye Research, Osaka, Japan, p. 51.

Church, R. L., and Tanzer, M. L. (1975). *FEBS. Lett.* **53,** 105–109.

Church, R. L., Yaeger, J. A., and Tanzer, M. L. (1974). *J. Mol. Biol.* **86,** 785–799.

Church, R. L., SundarRaj, N., and McDougall, J. K. (1980). *Cytogenet. Cell. Genet.* **27,** 24–30.

Compans, R. W., Holmes, K. V., Dales, S., and Choppin, P. W. (1964). *Virology* **30,** 411–426.

Cramp, F. C., and Lucy, J. A. (1974). *Exp. Cell Res.* **87,** 107–110.

Creagan, R. P., and Ruddle, F. H. (1977). *In* "Molecular Structure of Human Chromosomes" (J. J. Yumis, ed.), pp. 90–142. Academic Press, New York.

Creagan, R. P., Chen, S., and Ruddle, F. H. (1975). *Proc. Natl. Acad. Sci. U.S.A.* **72,** 2237–2241.

Croce, C. M., and Koprowski, H. (1974). *J. Exp. Med.* **139,** 1350–1353.
Croce, C. M., and Koprowski, H. (1975). *Proc. Natl. Acad. Sci. U.S.A.* **72,** 1658–1660.
Croce, C. M., Sawicki, W., Kritchewsky, D., and Koprowski, H. (1971). *Exp. Cell Res.* **67,** 427–435.
Davidson, R. L., and Gerald, P. S. (1976). *Somatic Cell Genet.* **2,** 165–176.
Diacumakos, E. G. (1973). *Proc. Natl. Acad. Sci. U.S.A.* **70,** 3382–3386.
Diacumakos, E. G., and Tatum, E. L. (1972). *Proc. Natl. Acad. Sci. U.S.A.* **69,** 2959–2962.
Drets, M. E., and Shaw, M. W. (1971). *Proc. Natl. Acad. Sci. U.S.A.* **68,** 2073–2077.
Ege, T., and Ringertz, N. R. (1974). *Exp. Cell Res.* **87,** 378–382.
Ege, T., Ringertz, N. R., Hamberg, H., and Sidebottom, E. (1977). *Meth. Cell Biol.* **15,** 339–357.
Elsevier, S. M., Kucherlopoti, R. S., Nichols, E. A., Creagan, R. P., Giles, R. E., Ruddle, F. H., Willecke, K., and McDougall, J. K. (1974). *Nature (London)* **251,** 633–636.
Ephrussi, B., and Weiss, M. C. (1965). *Proc. Natl. Acad. Sci. U.S.A.* **53,** 1040–1042.
Eun, C. K., and Klinger, H. P. (1979). *Cytogenet. Cell Genet.* **25,** 151.
Eun, C. K., and Klinger, H. P. (1980). *Cytogenet. Cell Genet.* **27,** 57–65.
Fournier, R. E. K., and Ruddle, F. H. (1977a). *Proc. Natl. Acad. Sci. U.S.A.* **74,** 319–323.
Fournier, R. E. K., and Ruddle, F. H. (1977b). *Proc. Natl. Acad. Sci. U.S.A.* **74,** 3937–3941.
Friedrich, U., and Coffino, P. (1977). *Proc. Natl. Acad. Sci. U.S.A.* **74,** 679–683.
Friend, K. K., Chen, S., and Ruddle, F. H. (1976a). *Somatic Cell Genet.* **2,** 183–188.
Friend, K. K., Dorman, B. P., Kucherlapati, R. S., and Ruddle, F. H. (1976b). *Exp. Cell Res.* **99,** 31–36.
Gefter, M. L., Margulies, D. H., Schorff, M. D. (1977). *Somatic Cell Genet.* **3,** 231–236.
Gershon, D., and Sachs, L. (1963). *Nature (London)* **198,** 912–913.
Giles, R. E., and Ruddle, F. H. (1973). *In* "Tissue Culture, Methods and Applications" (P. F. Kruse, Jr., and M. K. Patterson, Jr., eds.), pp. 475–500. Academic Press, New York.
Green, E. L. (1975). "Biology of the Laboratory Mouse." 2nd ed. Dover, New York.
Guggenheim, M. A., Friedman, R. M., and Rabson, A. S. (1968). *Science* **159,** 542–543.
Hales, A. (1977). *Somatic Cell Genet.* **3,** 227–230.
Handmaker, S. D. (1973). *Annu. Rev. Microbiol.* **27,** 189–204.
Hansen, D., and Stadler, J. (1977) *Somatic Cell Genet.* **3,** 471–482.
Harris, H., and Hopkinson, D. A. (1976). "Handbook of Enzyme Electrophoresis in Human Genetics." Elsevier, Amsterdam.
Harris, H., and Watkins, J. F. (1965). *Nature (London)* **205,** 640–646.
Harris, J., and Whitmore, G. (1974). *J. Cell. Physiol.* **83,** 43–49.
Hay, E. D., Linsenmayer, T. F., Trelstad, R. L., and Von der Mark, K. (1979). *In* "Current Topics in Eye Research," (J. A. Zadunaisky and H. Davson, eds.), pp. 1–36. Academic Press, New York.
Hecht, T. T., Ruddle, N. H., and Ruddle, F. H. (1975). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **34,** 995.
Henle, G., Deinhardt, F., and Girardi, A. (1954). *Proc. Soc. Exp. Biol. Med.* **87,** 386–393.
Hilwig, I., and Gropp, A. (1972). *Exp. Cell Res.* **75,** 122–126.
Hitchcock, G. (1971). *Exp. Cell. Res.* **67,** 463–466.
Hoggan, M. D., and Roizman, B. (1959). *Am. J. Hyg.* **70,** 208–219.

Howe, C., Morgan, C., deVaux St. Cyr, C., Hsw, K. C., and Rose, H. M. (1967). *J. Virol.* **1,** 215–257.
Hsie, A., Brimer, P., Mitchell, T., and Gosslee, D. (1975). *Somatic Cell Genet.* **1,** 247–269.
Hynes, R. O. (1976). *Biochim. Biophys. Acta.* **458,** 73–107.
Johnson, R. T., Mullinger, A. M., and Skaer, R. J. (1975). *Proc. R. Soc. London, Ser. B* **189,** 591–602.
Jones, C., Wuthier, P., and Puck, T. T. (1975). *Somatic Cell Genet.* **1,** 235, 246.
Kao, K. N., and Michayluck, M. R. (1974). *Planta* **115,** 355–367.
Kefalides, N. A. (1979). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **38,** 816.
Keller, W. A., and Melchers, G. (1973). *Z. Naturforsch., C: Biochem. Biophys. Biol. Virol.* **28,** 737–741.
Klebe, R. J. (1974). *Nature (London)* **250,** 248–251.
Klebe, R. J., Chen, T. R., and Ruddle, F. H. (1970). *J. Cell Biol.* **45,** 74–82.
Klobutcher, L. A., and Ruddle, F. H. (1979). *Nature (London)* **280,** 657–660.
Kohn, A. (1965). *Virology* **26,** 228–245.
Kucherlapati, R. S., Hilwig, I., Gropp, A., and Ruddle, F. H. (1975). *Humangenetik* **27,** 9–14.
Kusano, T., Long, C., and Green, H. (1971). *Proc. Natl. Acad. Sci. U.S.A.* **68,** 82–86.
Levan, A., Fredga, K., and Sandberg, A. A. (1964). *Hereditas* **52,** 201–220.
Littlefield, J. W. (1964) *Science* **145,** 709–710.
McBride, O. W., and Ozer, H. L. (1973). *Proc. Natl. Acad. Sci. U.S.A.* **70,** 1258–1262.
McDonald, T. F., Sachs, H. G., Orr, C. W. M., and Ebert, J. D. (1972). *Exp. Cell Res.* **74,** 201–208.
McDougall, J. K. (1971) *J. Gen. Virol.* **12,** 43–51.
McDougall, J. K., Kuchlerlapti, R., and Ruddle, F. H. (1973). *Nature (London) New Biol.* **245,** 1972–1975.
McKusick, V. A. (1972) "Heritable Disorders of Connective Tissue" 4th ed. C. V. Mosby, St. Louis.
Maio, J. J., and Schildkraut, C. L. (1967). *J. Mol. Biol.* **24,** 29–39.
Mankovitz, R., Baker, R. M., and Buchwald, M. (1973) *13th Ann. Meet. American Soc. of Cell Biol.,* Miami Beach p. 214a.
Mayhew, E., and Levinson, C. (1968). *J. Cell. Physiol.* **72,** 73–80.
Medrano, L., and Green, H. (1974). *Cell* **1,** 23–26.
Migeon, B. R., and Miller, C. S. (1968). *Science* **162,** 1005–1006.
Miller, O. J., Allderdice, P. W., Miller, D. A., Breg, W. R., and Migeon, B. R. (1971). *Science* **173,** 244–245.
Minor, R. R. (1980). *Am. J. Pathol.* **98,** 226–280.
Mintz, B. (1971). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **30,** 935–943.
Mintz, B., and Baker, W. W. (1967). *Proc. Natl. Acad. Sci. U.S.A.* **58,** 592–598.
Moses, E., and Kohn, A. (1963) *Exp. Cell Res.* **32,** 182–186.
Mukherjee, A. B., Orloff, S., De B. Butler, J., Triche, T., Lalley, P., and Schulman, J. D. (1978). *Proc. Natl. Acad. Sci. U.S.A.* **75,** 1361–1365.
Norwood, T. H., Zeigler, C. J., and Martin, G. M. (1976). *Somatic Cell Genet.* **2,** 263–270.
Okada, Y. (1958). *Biken's J.* **1,** 103–110.
Okada, Y. (1969). *Curr. Top. Microbiol. Immunol.* **48,** 102–128.
Okada, Y., and Murayama, F. (1965). *Exp. Cell Res.* **40,** 154–158.
Owerbach, D., Doyle, D., and Shows, T. B. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75,** 5640–5644.
Papahadjopoulos, D., Poste, G., and Schaeffer, B. E. (1973). *Biochim. Biophys. Acta.* **323,** 23–42.

Papahadjopoulos, D., Mayhew, E., Poste, G., Smith, S., and Vail, W. J. (1974). *Nature (London)* **252,** 163–166.
Paris Conference, 1971 (1972). *Birth Defects Orig. Artic. Ser.* **VIII**(7), 1–46.
Park, E. D., Church, R. L., and Tanzer, M. L. (1975) *Immunology* **28,** 781–790.
Pontecorvo, G. (1975). *Somatic Cell Genet.* **1,** 397–400.
Poole, A. R., Howell, J. I., and Lucy, J. A. (1970). *Nature (London)* **227,** 810–814.
Poste, G. (1970). *Adv. Virus Res.* **16,** 303–356.
Poste, G. (1972). *Exp. Cell Res.* **73,** 273–286.
Poste, G. (1973). *Methods Cell Biol.* **7,** 211–250.
Prescott, D. M., Myerson, D., and Wallace, J. (1972). *Exp. Cell Res.* **71,** 480–485.
Rao, P. N., and Johnson, R. T. (1972). *Methods Cell Physiol.* **5,** 75–126.
Rennard, S. I., Church, R. L., Rohrbach, D. H., Shupp, D. E., Abe, S., Hewitt, A. T., Murray, J. C., and Martin, G. R. (1981). *Biochem. Genet.,* in press.
Ringertz, N. R., and Savage, R. E. (1976). "Cell Hybrids." Academic Press, New York.
Ruddle, F. H., and McBride, O. W. (1977). *In* "The Molecular Biology of the Mammalian Genetic Apparatus" (P. Ts'o, ed.) Vol. 2, pp. 163–169. Elsevier, Amsterdam.
Sanchez, O., and Yunis, J. J. (1977). *In* "New Chromosomal Syndromes" (J. J. Yunis, ed.), pp. 1–54. Academic Press, New York.
Sato, K., Slesinski, R., and Littlefield, J. (1972). *Proc. Natl. Acad. Sci. U.S.A.* **69,** 1244–1248.
Schnedl, W. (1971). *Nature (London) New Biol.* **233,** 93–94.
Schnedl, W. (1974a). *In* "Methods in Human Cytogenetics" (H. G. Schwarzacher, U. Wolf, and E. Passarge, eds.), pp. 95–118. Springer-Verlag, Berlin and New York.
Schnedl, W. (1974b). *Int. Rev. Cytol., Suppl.* **4,** 237–272.
Schor, S. L., Johnson, R. T., and Mullinger, A. M. (1975). *J. Cell Sci.* **19,** 281–303.
Seabright, M. (1971). *Lancet* **2,** 971–972.
Shows, T. B., and McAlpine, P. J. (1979). *Cytogenet. Cell Genet.* **25,** 117–127.
Siminovitch, L. (1976). *Cell* **7,** 1–11.
Smith, M., Gold, L. I., Pearlstein, E., and Krinsky, A. (1979). *Cytogenet. Cell Genet.* **25,** 205.
Solomon, E., and Sykes, B. (1979). *Cytogenet. Cell Genet.* **25,** 205.
Sorieul, S., and Ephrussi, B. (1961). *Nature* **190,** 653–654.
Spandidos, D. A., and Siminovitch, L. (1977a). *Proc. Natl. Acad. Sci. U.S.A.* **74,** 3480–3484.
Spandidos, D. A., and Siminovitch, L. (1977b). *Cell* **12,** 235–242.
Steplewski, Z., Koprowski, H., and Leibovitz, A. (1976). *Somatic Cell Genet.* **2,** 559–564.
Sumner, A. T., Evans, H. J., and Buckland, R. A. (1971). *Nature (London) New Biol.* **232,** 31–32.
Sundar-Raj, C. V., Church, R. L., Creagan, R. P., and Ruddle, F. H. (1976). *J. Cell Biol.* **70,** 78a.
Sundar-Raj, C. V., Church, R. L., Klobutcher, L. A., and Ruddle, F. H. (1977). *Proc. Natl. Acad. Sci. U.S.A.* **74,** 4444–4448.
Sykes, B., and Solomon, E. (1978). *Nature (London)* **272,** 548–549.
Sykes, B., Puddle, B., Francis, M., and Smith, R. (1976). *Biophys. Biochem. Res. Commun.* **72,** 1472–1480.
Szybalski, W., Szybalski, E. H., and Ragni, G. (1962). *Natl. Cancer Inst. Monogr.* **7,** 75–89.
Tan, Y. H., Tischfield, J. A., and Ruddle, F. H. (1973). *J. Exp. Med.* **137,** 317–330.
Thirion, J.-P., Banville, D., and Noël, H. (1976). *Genetics* **83,** 137–147.
Tischfield, J. A., and Ruddle, F. H. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71,** 45–49.

Toister, Z., and Loyter, A. (1971). *Biochim. Biophys. Acta* **241,** 719–724.
Toister, Z., and Loyter, A. (1973). *J. Biol. Chem.* **248,** 422–432.
Vaheri, A., and Ruoslahti, E. (1975). *J. Exp. Med.* **142,** 530–535.
Wahl, G., Hughes, S., and Capecchi, M. (1975). *J. Cell Physiol.* **85,** 307–314.
Watkins, J. F. (1971). *Int. Rev. Exp. Pathol.* **10,** 115–141.
Weiss, M. C., and Green, H. (1967). *Proc. Natl. Acad. Sci. U.S.A.* **58,** 1104–1111.
Willecke, K., and Ruddle, F. H. (1975). *Proc. Natl. Acad. Sci. U.S.A.* **72,** 1792–1796.
Willecke, K., and Lange, R., Krüger, A., and Reber, T. (1976). *Proc. Natl. Acad. Sci. U.S.A.* **73,** 1274–1278.
Wright, W. E., and Hayflick, L. (1972). *Exp. Cell Res.* **74,** 187–194.
Wullems, G. J., Van der Horst, J., and Bootsma, D. (1975). *Somatic Cell Genet.* **1,** 137–152.
Wullems, G. J., Van der Horst, J., and Bootsma, D. (1976a). *Somatic Cell Genet.* **2,** 155–164.
Wullems, G. J., Van der Horst, J., and Bootsma, D. (1976b). *Somatic Cell Genet.* **2,** 359–371.
Wullems, G. J., Van der Horst, J., and Bootsma, D. (1977). *Somatic Cell Genet.* **3,** 281–294.
Yamada, K. A., and Olden, K. (1978) *Nature (London)* **275,** 179–184.
Yanovsky, A., and Loyter, A. (1972) *J. Biol. Chem.* **247,** 4021–4028.
Yerganian, G., and Nell, M. B. (1966). *Proc. Natl. Acad. Sci. U.S.A.* **55,** 1066–1073.