PRODUCTION OF HUMAN-MOUSE SOMATIC CELL HYBRIDS AND CHARACTERIZATION FOR CARCINOEMBRYONIC ANTIGEN EXPRESSION

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BY

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A thesis submitted to the School of Graduate Studies in partial fulfillment of the requirements for the degree of Master of Science

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April 1987

St. John's Newfoundland

ISBN 0-315-39488-9

ABSTRACT

Carcinoembryonic antigen (CEA), a human tumor-associated marker for many epithelial tumors, has been well characterized immunologically and structurally but its biological function(s) remains a mystery. The objectives of this investigation were to produce interspecific somatic cell hybrids between human colorectal, CEA expressing/secreting, cancer cell lines (LS174T, SKCO1 and HT29) and mouse cell lines (NS1, SP2/0, RAG, PG19 and STO) and to characterize the resultant hybrids for CEA expression/secretion with the longer term goal of assigning the CEA gene(s) to a particular chromosome.

Fusions were performed using a polyethylene glycol (PEG) suspension technique and hybrids were isolated by hypoxanthine-aminopterin-thymidine/ouabain double selection and characterized for CEA expression/secretion in specific CEA immunoassays. Forty-eight fusions were performed (43, monolayer-suspension; 5, monolayer-monolayer) producing 344 hybrid colonies of the following types (SKCO1xRAG, SKCO1xSTO, HT29xRAG, HT29xSTO and HT29xNS1). Chromosomal analysis by Giemsa differential staining confirmed that these fusion products were definite hybrids containing human and mouse chromosomes.

This study has demonstrated (1) the successful production of human-mouse somatic cell hybrids, (2) that fusion of monolayer-monolayer cell lines resulted in a greater yield of hybrids and (3) that none of the hybrids obtained showed high levels of CEA expression/secretion, probably because the RAG and STO cell lines were nonpermissive fusion partners.

ACKNOWLEDGEMENTS

I am indebted to everyone who offered advice and encouragement during the entire M.Sc. program. I am especially indebted to Dr C.H.J. Ford for his undying support and direction throughout the project. It took a special supervisor to show me that good things do not come without hard work. This last two and one half years have provided me with a list of unforgettable experiences which I am sure will benefit me in my future vocation. In addition, I wish to thank both Dr. K. Ali, for his advice and encouragement, and the rest of the Oncology Research group (Vernon, Maureen, Gen, Sylvia, Alan, Vijay and Zetta) for providing the lighter side of basic research to me. For technical advice and assistance in the chromosome methods, I wish to thank Sylvia, Debbie and Diana, and the Cytogenetics Laboratory at the Janeway (Olga, Tom, Debbie and Lorraine). For performing the majority of the immunocytochemical analysis, I wish to thank Mrs. Maureen Gallant. For helping in the typing of my thesis, I wish to thank Mrs. Gen Wight. For advice and direction during the write-up of my thesis, I wish to thank my supervisory committee: Dr. Verna Skanes, Dr. Penny Allderdice and Dr. Clarke Fraser. For financial support I am indebted to the Cancer Research Society and Graduate Studies in the Faculty of Medicine. Finally, I wish to thank my wife, Nicole, for her endless concern and motivation, and my family for their support throughout this degree program.

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LIST OF ABBREVIATIONS

ABTS	2,2'-azino-di(3-ethylbenzthiazoline sulfonic acid)
AFP	alpha fetoprotein
8-AG	8-azaguanine
BGP	biliary glycoprotein
5-Brdu	5-bromodeoxyuridine
CEA	carcinoembryonic antigen
CELIA	gastric CEA-like antigen
DMGT	DNA mediated gene transfer
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetate acid
ELISA	enzyme linked immunosorbent assay
FCS	fetal calf serum
G-banding	Giemsa banding
G-11	Giemsa alkaline
HAT	hypoxanthine-aminopterin-thymidine
HGPRT	hypoxanthine-guanine phosphoribosyl transferase
KD	kilodalton
NCA	nonspecific cross-reacting antigen
PBS	phosphate buffered saline
PCA	perchloric acid
PEG	polyethylene glycol
RFLPs	restriction fragment length polymorphisms
RNA	ribonucleic acid
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis

LIST OF ABBREVIATIONS (continued)

TEX	tumor extracted antigen
6-TG	6-thioguanine
TK	thymidine kinase

Chapter 1

INTRODUCTION

1.1 Gene Mapping

1.1.1 Introduction

Gregor Mendel, the father of classical genetics, formulated the laws of inheritance and inferred from these laws that individual traits were passed on from generation to generation by "factors" in the cell. "Factors" was the term used by Mendel for the entities that were later designated "genes" by Johanssen in 1909. By the early 1900's, evidence was compiled supporting the hypothesis that the primary components of the cell nucleus, the chromosomes, carried the genetic information for development and heredity. Since that time geneticists have been interested in knowing the location, arrangement, and linkage of individual genes; especially the genes responsible for inherited diseases (McKusick and Ruddle, 1977).

1.1.2. Methods of Gene Mapping

The three methods used in chromosome mapping are classical pedigree linkage analysis, somatic cell genetic analysis and molecular genetic analysis. By studying the inheritance pattern in families it was demonstrated that colorblindness was a sex linked recessive trait affecting males who contained the defective X chromosome and hence the first gene was mapped to a specific human chromosome (Wilson, 1911). Over the next fifty years only a few dozen other X-linked traits (e.g.,

hemophilia and Duchenne muscular dystrophy) were identified by characteristic pedigree pattern. In addition nine autosomal linkages were established, however, because of the lack of cytogenetic markers, the specific autosomes responsible for each phenotype could not be determined. The first assignment of a gene by family linkage analysis to a specific human autosome occurred in 1968 when Donahue and colleagues postulated that the Duffy blood group locus "Fy" was on chromosome 1 (Donahue, Bias, Renwick and McKusick, 1968). They made the assignment by finding evidence of linkage between the Duffy locus and a normal variation in chromosome 1 that was segregating in a mendelian manner in the family studied.

In the late 1950's to early 1960's three scientists independently suggested using "parasexual experimental systems" for the study of genes in eukaryotic organisms (Lederberg, 1958; Stern, 1958; Pontecorvo, 1962). It was around this time that the parasexual method of genetic study was introduced as an alternative method for assigning specific genes to specific chromosomes. The parasexual production of interspecific human-mouse hybrid cells by whole cell fusion of the parental cells was referred to as somatic cell hybridization. Some important developments leading up to the discovery that somatic cell hybridization could be used as a genetic mapping technique were as follows:

(1) The application of microbial genetic techniques to mammalian cells resulted in many kinds of genetic and biochemical studies. For example, the establishment of long term in vitro mammalian cell cultures and tissue culture techniques resulted

in many new genetic investigations (Puck, 1972).

(2) The first in vitro cell hybridization was demonstrated by mixing cultures of two mouse sarcoma cell lines and deriving a hybrid cell line (Barski, Sorieul and Cornefert, 1961). This spontaneous fusion of genetically different cell lines was confirmed by other researchers (Sorieul and Ephrussi, 1961; Gershon and Sachs, 1963).

(3) A hybrid selection system was developed by using selection medium containing hypoxanthine, aminopterin and thymidine (HAT medium) and conditionally lethal mutant parent cell lines (Littlefield, 1964).

(4) Virus fusion agents were shown to improve fusion efficiencies. For example, myxovirus was used as a fusing agent for mammalian somatic cells (Okada and Tadokoro, 1962). Other researchers confirmed this fusing ability by using ultraviolet light inactivated Sendai virus to produce hybrids between human HeLa cells and Ehrlich ascites tumor cells from mice. In addition this demonstrated the first interspecific hybridization (Harris and Watkins, 1965; Ephrussi and Weiss, 1965).

(5) The chromosome segregating property of human-mouse interspecific hybrids was identified when it was observed that human-mouse hybrids tended to lose human chromosomes (Weiss and Green, 1967).

The first assignment of a gene to a specific chromosome was achieved using somatic cell hybrid technology in 1971. By studying human-mouse hybrid mapping panels, containing various assortments of human chromosomes, the thymidine kinase (TK) gene was assigned to chromosome 17 (Miller, Allderdice and Miller, 1971). This was determined by correlating the presence or absence of a gene product (TK) with the presence or absence of a particular human chromosome (chromosome 17). In the 1970's the number of specific gene assignments was increased dramatically and by 1976 at least one gene had been assigned to each human chromosome. The development of characteristic banding patterns such as quinacrine-banding and Giemsa-banding improved the resolution of gene mapping (Caspersson, Zech, Johanssen and Modest, 1970; Seabright, 1971). At this time chromosome banding patterns tended to be of low resolution, but with the development of chromosome synchronization techniques, it was increased from 400 to 2000 bands (Yunis, 1976; Francke and Oliver, 1978).

Towards the end of the 1970's and the beginning of the 1980's molecular genetic methods were being applied to chromosome mapping. Some earlier important developments such as the discovery of restriction enzymes, the development of human cloned DNA libraries and the development of recombinant DNA techniques facilitated molecular genetic studies. One particular technique which has improved the resolution of gene mapping was developed in 1969 by Gall and Pardue. They demonstrated the location of the ribosomal genes in oocytes of the toad Xenopus by direct hybridization of radiolabelled RNA probes to the complementary DNA in a cytological preparation (Gall and Pardue, 1969). Today this technique

which is referred to as in situ hybridization is used to map genes to specific subregions on chromosomes by hybridizing radiolabelled or fluorescent labelled DNA probes directly to chromosome spreads. Besides the direct mapping technique of nucleic acid hybridization, molecular genetics provided new markers for family linkage studies. The new markers are human DNA polymorphisms or "restriction fragment length polymorphisms" (RFLPs). These RFLPs are clinically important as linkage markers for certain genetic diseases such as Duchenne muscular dystrophy, Huntington's disease, adult polycystic kidney disease and cystic fibrosis.

The human chromosome map now has information on the chromosomal location of more than 800 specific genes and information on the location of as many functionally unknown DNA segments. Some of the genes mapped include genes for enzymes, hormones, cell surface proteins, growth factors, complement and cellular oncogenes. The human chromosome map and current mapping strategies have recently been reviewed (McKusick,1986).

1.1.3 Chromosomes, Oncogenes and Cancer

Certain chromosomal defects are consistently associated with some types of human cancer. For example, in both retinoblastoma and Wilm's tumor, the tumor cells often exhibit a deletion of a particular chromosomal segment (13q14 for retinoblastoma; 11q13 for Wilm's tumor).

Along with chromosomal defects a group of genes referred to as "oncogenes" have been shown to be associated with certain cancers.

Oncogenes can be classified into two types depending on their host; viral and cellular. In the case of cellular oncogenes (c-oncs) the corresponding wild-type allele is called the proto-oncogene. One such proto-oncogene that has been well studied is c-myc. C-myc, originally found in B-cell avian myelocytoma and assigned to band q24 in chromosome 8 by in situ hybridization (Neel, Jhanwar, Chaganti and Hayward, 1982), is associated with Burkitt's lymphoma (Taub, Kirsch, Morton, Lenoir, Swan, Tronick, Aaronson and Leder, 1982). The common defect observed is a reciprocal translocation involving c-myc and the immunoglobulin heavy chain genes on chromosome 14. In some cases the translocation occurs between c-myc and the immunoglobulin light chain genes (chromosomes 2 or 22)(Yunis, 1983). Another oncogene, tcl-1 (T-cell lymphoma/leukemia-1) was proposed by Croce and colleagues to be located on band q32.3 of chromosome 14 and activated in various T-cell malignancies. It appears that the α-chain gene of the T cell receptor, which was localized to 14q11-12 by in situ hybridization, may be involved in oncogene activation following chromosomal translocations or inversions in T cells (Croce, Isobe, Palumbo, Puck, Ming, Tweardy and Erikson, 1985). A second oncogene, tcl-2, was proposed to reside within 11p13 and be involved in T-cell malignancies in a similar manner to the tcl-1 oncogene (Erikson, Williams, Finan, Nowell and Croce, 1985).

To date more than 30 oncogenes have been identified and 28 of these have been mapped specifically to human chromosomes (Table 1). Most of the mapping assignments have been achieved with the combination of both somatic cell and molecular genetic techniques. For example, the c-sis and c-Ki-ras2 oncogenes were localized to their specific chromosomes (22 for c-sis; 12 for c-Ki-ras2) by Southern hybridization analysis of human-rodent somatic cell hybrids with molecular genetic probes to the oncogenes (Dalla-Favera, Gallo, Giallongo and Croce, 1982; Sakaguchi, Naylor, Shows, Toole, McCoy and Weinberg, 1983). Direct confirmation of these assignments is usually achieved with <u>in situ</u> hybridization analysis of hybrid metaphase cells.

Some oncogenes code for protein kinases, growth factors and growth factor receptors and one proposal is that they are mitosis regulatory genes in which mutation can result in loss of growth control and production of a cancerous cell (Gordon, 1985).

Table 1 Oncogenes mapped to specific human chromosomes.*

Function of the oncogene product	Oncogene	Location in the human karyotype
1. Protein kinase	src	1p34-pter and 20q12-q13
	abl	9q34
	fes	15q25-q26
	yes	18
2. Guanosine triphosphate binding	H-ras-1	11p15.1-p15.5
	H-ras-2	X
	K-ras-1	6p23-q12
	K-ras-2	12p12-pter
	N-ras	1p22-p31
3. DNA binding	myc	8q24
	N-myc	2p23-p24
	myb	6q15-q24
	ets	11q23-q24
	ski	1q12-qter
	fos	16
4. Growth factor	sis	22q12.3-q13.1
5. Growth factor receptors	erb-B	7p1-p21
	fms	5q3
	neu	17
	mos	8q22
6. Uncertain	erb-A	17p11-q21
	rel	2p11-q14
	Blym	1p32

* Modified version of a table in H. Gordon's review of oncogenes (Gordon, 1985).

1.2 Somatic Cell Hybridization

1.2.1 Mapping Genes for Cell Surface Antigens

The technique of somatic cell hybridization has permitted a rapid acceleration in defining the human gene map since 1971. In the early 1970's most gene assignments determined by studying human-rodent hybrid cells were primarily restricted to enzyme coding genes. The reason was that most human and rodent isoenzymes could be distinguished by their electrophoretic patterns. In addition, another group of genetic markers, human specific antigens, were mapped by determining their expression/nonexpression in a panel of human-rodent hybrids. At first, conventional polyclonal antisera were used for assessing antigen expression in hybrids. Soon after, with the development of monoclonal antibody technology (Kohler and Milstein, 1975), monoclonal antibodies specific for cell surface antigens were used because of the advantages they offered over polyclonal antisera. Some of these advantages include: (1) standardization of reagents, (2) reproducibility, (3) unlimited availability of reagents and (4) immunochemical purity of reagents. Illustrated in Table 2 are some cell surface antigen/receptor gene assignments which have been characterized with monoclonal antibodies specific for each antigen.

Table 2. Cell surface antigens/receptors mapped by characterization of somatic cell hybrids with monoclonal antibodies.

Cell Surface Antigen	Location in the human karyotype	Reference
1. Human p53 cellular tumor antigen	17	McBride, Merry and Givol, 1986.
2. Human T-cell receptor alpha chain	14	Croce, Isobe, Palumbo, Puck, Ming, Tweardy and Erikson, 1985.
3. Human Transferrin receptor	3	Goodfellow, Banting, Sutherland, Greaves, Solomon and Povey, 1982.
4. Human Insulin receptor	19	Yang-Feng, Francke and Ullrich, 1985.
5. Human chromosome one cell surface markers	1	Rettig, Dracopoli, Goetzger, Spengler, Biedler, Oettgen and Old, 1984.
6. T4 Antigen on subset of T cells	12	Kozbor, Finan, Nowell and Croce, 1986.

1.2.2 Selection Systems

The first hybrid cells were isolated without any selection against unfused parental cells. The hybrids outgrew both parental cell types (Barski et al., 1961; Davidson and Ephrussi, 1965; Yerganian and Nell, 1966). Illustrated in Table 3 are some of the hybrid selection systems that have been used since the development of somatic cell hybridization.

Table 3. Systems used for hybrid selection.

Selection System	Hybrid Selection Factor	Reference
1. Natural selection	Hybrid vigor	Barski et al., 1961
2. Visual selection	a) Different cell morphology b) Larger nucleus and larger cell	Weiss and Green, 1967
3. Selection by genetic markers	a) Enzyme deficient parent cells	Szybalski, Szybalski and Ragni, 1962;
	b) Temperature sensitive parent cells	Goldstein and Lin, 1972
4. Selection by virus infection	Human cytopathogenic viruses	Zepp, Conover, Hirschhorn and Hodes, 1971

Selection systems play an important role in the isolation of somatic cell hybrids between human and rodent cells. Most selective systems depend on acquired metabolic properties such as drug resistance, auxotrophy or differential sensitivity. The concept of selection systems has been reviewed by Chu and Powell (1976).

1.2.3 The Hypoxanthine-Aminopterin-Thymidine/Ouabain Selection System

Both Szybalski and Littlefield independently reported the first use of selection medium allowing the growth of hybrid cells but resulting in death of the two parental cell types (Szybalski et al., 1962; Littlefield, 1964). This selection is based on the use of HAT medium, which was devised by Szybalski to kill cells that lack hypoxanthine-guanine phosphoribosyl transferase (HGPRT) or thymidine kinase (TK). Aminopterin inhibits dihydrofolate reductase and blocks de novo synthesis of purines and pyrimidines. This selectively pressures the cells to synthesize nucleotides via the salvage pathway. To utilize the salvage pathway the preformed bases, hypoxanthine and thymidine, must be present in the growth medium. The parent cell lines are classified as "conditionally lethal mutants" because of their enzyme deficiencies. In order to develop these enzyme deficient cell lines the wild type cells must be made: (1) drug resistant to purine analogues such as 8-azaquanine (8-AG) or 6-thioguanine (6-TG) for HGPRT deficiency or (2) drug resistant to pyrimidine analogues such as 5-bromodeoxyuridine (5-Brdu) for TK deficiency. When the wild type cells are grown in the presence of the analogues, selective pressures result in the mutation of the genes coding for the salvage pathway enzymes. This defect in the enzymes is reflected by drug resistance to the nucleotide analogues.

Another selection agent commonly used in interspecific hybridization of human and mouse cells is the drug "ouabain". Ouabain, or strophanthin, is a cardiac glycoside which inhibits the Na^+/K^+ activated ATPase of the plasma membrane, the enzyme responsible for the active transport

of K^+ into the cell and the extrusion of Na^+. One parent cell line lacks either HGPRT or TK activity whereas the other parent cell line is sensitive to the concentration of ouabain used in the medium. Since human cells are usually killed by low concentrations of ouabain (approx. 10^{-6}M) and mouse cells by higher concentrations of ouabain (approx. 10^{-3}M), this drug is often used for selecting human-mouse hybrids. Illustrated in Table 4 are the common chemical selection systems used for the isolation of hybrid cells from their conditionally lethal parent cells.

Table 4. Common chemical selection systems used in somatic cell hybridization.

Selection Medium	Enzyme defect	Reference
1. Hypoxanthine-aminopterin-thymidine (HAT) selection	HGPRT or TK	Littlefield, 1964.
2. Adenine-alanosine (AA) selection	APRT	Kusano, Long and Green, 1971.
3. Hypoxanthine-aminopterin-5-methyldeoxycytidine (HAM) selection	HGPRT, TK, and dCD	Chan, Long and Green, 1975.
4. Deoxycytidine-thymidine (dCR-dTR) selection	dCK	De Saint Vincent and Buttin, 1973.
5. HAT-ouabain selection	HGPRT or TK	Kucherlapati, Baker and Ruddle, 1975.

APRT, adenine phosphoribosyl transferase; dCD, deoxycytidine deaminase; dCK, deoxycytidine kinase.

Initial fusion experiments depended on spontaneous fusion of the parent cell types for the development of hybrids. The fusion efficiency has been improved by the incorporation of fusion agents into the fusion technique. Illustrated in Table 5 are some of the common fusion agents used in somatic cell fusions.

Table 5. Different fusing methods used for the production of hybrids.

Fusion technique	Fusing agent	Reference
1. Virus mediated cell fusion	UV-inactivated Sendai virus	Okadi, 1962
2. Polyethylene glycol mediated cell fusion	Polyethylene glycol	Pontecorvo, 1975
3. Electrically induced cell fusion	Electricity	Finaz, Lefevre and Teissie, 1984

The applications of cell fusion studies include genetic analysis of somatic cells, regulation of gene expression, control of malignancy, virus-cell interaction and gene mapping. For the most of the 1970's the main source of information for chromosomal assignment of genes was cell fusion studies, supplemented by family linkage studies. By 1979, molecular genetic studies (in situ hybridization) were also making significant contributions to gene mapping.

1.3 Cancer Markers

Since the hypothesis of the German biologist, T. Boveri, that the key factor initiating neoplastic transformation was the presence of abnormal chromosomes (Boveri, 1914; Verma, 1986), many other ideas concerning the etiology of cancer have developed. At the time of Boveri's hypothesis, tumor viruses were implicated as the causative factors. In the 1930's and 1940's chemicals and radiation were linked to the promotion of cancers. Today, many factors have been implicated in the transformation of a normal cell to an uncontrolled proliferating cancer cell (Littefield, 1984). According to the "somatic mutation hypothesis", the uncontrolled growth illustrated in cancers is the result of some genetic alteration in the cellular genome. This change, which may be observable as a chromosome abnormality, can be precipitated by predisposing genetic factors or environmental factors.

One theory concerning the switch from a normal cell to a cancerous cell postulates that genetic mutation of regulatory gene(s) controlling cell growth and differentiation is the primary event responsible for oncogenesis. That is, if normal regulatory processes are repressed or altered by some genetic variation, the normal cell may progress to uncontrolled cell growth. The stage of differentiation at which this change occurs results in expression of "differentiation antigens". Of the three basic classes of potential tumor markers (ie. hormones,

enzymes, and tumor associated antigens) the tumor associated antigens have been postulated as being differentiation specific antigens. The two most extensively characterized tumor antigens, carcinoembryonic antigen (CEA) and alpha fetoprotein (AFP) are closely related to differentiation and cancer. Both are classified as "oncofetal antigens" because they are markers associated with normal fetal development and a variety of malignant and non-malignant diseases.

1.3.1 Alpha Fetoprotein (AFP)

AFP is a 70 KD molecular weight serum protein expressed by fetal liver cells and primary hepatomas. This major fetal protein was first demonstrated in rodent hepatomas and, subsequently, in human hepatomas (Abelev, Perova, Khramkova, Postnikova and Irlin, 1963; Tatarinov, 1964). In addition, other studies indicated AFP is increased in other pathological defects such as germ cell tumors (Alpert, 1972), hereditary tyrosinemia (Belanger, 1973), neural tube defects (Allan, Ferguson-Smith, Donald, Sweet and Gibson, 1973; Brock and Sutcliffe, 1973) and ataxia telangiectasia (Waldman and McIntire, 1972). Hereditary tyrosinemia is an inborn metabolic disease characterized by abnormal tyrosine and methionine metabolism while ataxia telangiectasia is an autosomal recessive disorder characterized by a defect in tissue differentiation of gut associated organs (thymus, liver) and by defective DNA repair.

AFP is the only oncofetal antigen that has been extensively studied in terms of its genetics. Several laboratories, using recombinant DNA techniques have developed human cDNA clones from human AFP mRNA extracted

from fetal livers (Beattie and Dugaiczyk, 1982) and from testicular embryonal carcinoma (Tamaoki, Morinaga, Sakai, Protheroe and Urano, 1983). Nucleotide sequencing of these probes revealed homology with human serum albumin and mapping, by in situ hybridization using radiolabelled probes for both genes, localized the genes to bands q11-22 of chromosome four (Harper and Dugaiczyk, 1983; Minghetti, Harper, Alpert and Dugaicyzk, 1983).

1.3.2 Carcinoembryonic Antigen (CEA)

The term carcinoembryonic antigen was first used to describe a tumor specific glycoprotein found in fetal colonic tissue and adult colorectal tumors (Gold and Freedman, 1965a,b). Subsequent characterization revealed that CEA was associated with a variety of other solid tumor types and non-malignant diseases, particularly diseases of an inflammatory nature. In addition to the association with the diseased state, CEA and CEA-like substances were found in small amounts in normal colonic mucosa and in the serum, saliva, and feces of apparently normal individuals. Illustrated in Table 6 is the distribution of CEA in various normal and clinical conditions.

Table 6. Reported CEA associations.

A.	Malignant diseases	Reference
	1. Colonic tumors	Gold and Freedman, 1965a,b.
	2. Breast tumors	Santen, Collette and Franchimont, 1980.
	3. Ovarian tumors	Chism, Warner, Wells, Crewther, Hunt, Marchalonis and Fudenberg, 1977.
	4. Lung tumors	Vincent and Chu, 1973; Ford, Newman and Lakin, 1977; De Young and Ashman, 1978
	5. Medullary thyroid tumors	Ishikawa and Hamada, 1976.

B.	Non-malignant diseases	Reference
	1. Inflammatory bowel disease	Moore, Kantrowitz and Zamchek, 1972a; Booth, King, Leonard and Dykes, 1974; Thompson, Gillies, Silver, Shuster, Freedman and Gold, 1974.
	2. Pancreatitis	Delwiche, Zamchek and Marcon, 1973.
	3. Rectal polyps	Doos, Wolff, Shinya, Dechabon, Stenger, Gottlier and Zamchek, 1975.
	4. Chronic bronchitis	Laurence, Stevens, Bettelheim, Darcy, Leese, Turberville, Alexander, Johns and Neville, 1972.
	5. Cholecystitis	Martin, Kibbey, Divecchia, Anderson, Catalano and Minton, 1976.

Table 6. (cont'd)

B. Non-malignant diseases	Reference
6. Cystic fibrosis	Davidson, Mincey, Israels and Wilcox, 1973; Wu, Herbst and Bray, 1976.
7. Alcoholic cirrhosis	Moore, Dhar, Zamchek, Keeley, Gottlier and Kupchik, 1972b; Khoo, Warner, Lie and Mackay, 1973.

C. Normal conditions	Reference
1. Heavy smoking	Steven and McKay, 1973; Alexander, Silverman and Chretien, 1976.
2. Normal colonic mucosa serum, saliva, feces, and colonic lavages	Chu, Reyneso and Hansen, 1972; Martin and Devant, 1973; Egan, Pritchard, Todd and Go, 1977.

1.3.3 Physicochemical Characterization of CEA.

CEA is a glycoprotein, usually found in the cellular membrane, with a molecular weight range of 180-200 KD as determined by both sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and molecular sieving (Krupey, Gold and Freedman, 1968; Slayter and Coligan, 1975). On ultracentrifugation it displays a single peak with a sedimentation constant of 6.2-6.8S (Coligan, Lautenschleger, Egan and Todd, 1972). On immunoelectrophoresis it exhibits β-electrophoretic mobility, has an isoelectric point of 3 to 4 (Coligan, Henkart, Todd and Terry, 1973; Banjo, Shuster and Gold, 1974) and on electron microscopy

it appears to be a twisted rod-shaped molecule (Slayter and Coligan, 1975). Compositional analysis of the carbohydrate moiety, which represents approximately 50-60% of CEA, demonstrated that the major sugar residue is N-acetylglucosamine and the minor sugar residues are fucose, mannose, galactose, and sialic acid (Banjo, Gold, Freedman and Krupey, 1972; Banjo, Gold, Gehrke, Freedman and Krupey, 1974a; Banjo, Shuster and Gold, 1974b). Both amino acid and carbohydrate sequencing have been limited because of the extensive glycosylation of CEA. Up to 1983 only the first 24 amino acids on the NH_2 terminal end and some of the oligosaccaride chains had been sequenced (Terry, Henkart, Coligan and Todd, 1972; Chandrasekaren, Davila, Nixon, Goldfarb and Mendicino, 1983). Analysis of the protein substructure revealed a single polypeptide chain, ranging from 575 to 829 amino acids (Slayter and Coligan, 1975; Todd and Shively, 1978) , with 6 intrachain disulfide bonds (Westwood and Thomas, 1975).

1.3.4 Heterogeneity of CEA.

It is evident that CEA is heterogeneous based on its physicochemical properties. The inherent heterogeneity may, however, be the result of analysing an impure preparation of CEA. The usual source material for purifying CEA is liver metastases of colonic adenocarcinoma. The initial purification step utilizes perchloric acid (PCA) extraction which separates CEA, because of its high carbohydrate content, from the majority of other cellular proteins (Krupey, Gold and Freedman, 1967; Coligan et al., 1972; Hammarstrom, Svenberg and Sundblad, 1976). There

is no evidence to suggest that PCA destroys or modifies the native structure of CEA (Ashman and De Young, 1979; Koch and McPherson, 1980). The usual sequence of purification steps for CEA include PCA extraction, column chromatography on Sepharose 4B and Sephadex G-200, and preparative block electrophoresis on Sephadex G-25 (Krupey, Wilson, Freedman and Gold, 1972). Modifications to this purification step include using other extraction procedures such as neutral pH extraction (Eveleigh, 1974), 8M Urea (Kimball and Brattain, 1978), 3M KCl (Keep, Leake and Rogers, 1978), sodium dodecyl sulfate (Leung, Eshdat and Marchesi, 1977), or lithium diiodosalicylate (Rosai, Tillack and Marchesi, 1972). The use of monoclonal anti-CEA immunosorbent columns for CEA purification has recently been shown to be an improvement as it can result in a higher yield and is a much shorter purification process (Ford, MacDonald, Griffin, Life and Bartlett, 1987). Despite improved purification techniques, CEA still remains heterogeneous and, therefore, it has been suggested that the material operationally defined as CEA may actually consist of a family of related glycoproteins (Vrba, Alpert and Isselbacher, 1975; Alpert, 1978).

1.3.5 CEA-Like Glycoproteins.

In addition to putative isoantigens there are glycoproteins immunologically cross-reactive to CEA. The first glycoprotein reported to be similar to CEA was nonspecific cross-reactive antigen or NCA (Von Kleist, Chavanel and Burtin, 1972). NCA was present as a contaminant in PCA extracts with CEA. Other laboratories reported antigens cross-reactive

with CEA such as normal glycoprotein (Mach and Pusztaszeri, 1972), CEA-associated protein (Darcy, Turberville and James, 1973), colonic carcinoembryonic antigen-2 (Turberville, Darcy, Laurence, Johns and Neville, 1973) and colon carcinoma antigen-III (Newman, Petras, Georgiadis and Hansen, 1974). Further studies demonstrated that these glycoproteins represented one cross-reactive antigen that was later defined as normal cross reactive antigen-1 (NCA-1). In addition, other CEA-like glycoproteins, extracted from different tissues, have also been described (Table 7). It is believed that CEA and the cross-reactive antigens belong to one common gene family and to date no known biological functions have been demonstrated for any of these antigens.

Table 7. CEA cross-reactive antigens.

Cross-reactive Antigen	Location	Refernce
1. Nonspecific cross-reacting antigen (NCA)	Normal spleen or lung	von Kleist, Chavanal and Burtin, 1972.
2. Biliary glycoproteins I,II,and III. (BGPI,II,III)	Normal hepatic bile, Bile from obstructed or inflamed gall bladders	Svenberg, 1976.
3. Nonspecific cross-reacting antigen-2 (NCA-2)	Normal and colonic cancer feces	Burtin, Chavanal and Hirsh-Marie, 1973.
4. Tumor extracted antigen (TEX)	Liver metastases from colonic cancer	Kessler, Shively, Pritchard and Todd, 1978.
5. Gastric CEA-like antigen (CELIA)	Normal gastric juice	Vuento, Rouslahti, Pihko, Svenberg, Ihamaki and Siurala, 1976.

1.3.6 Genetics of CEA.

CEA is one of the best characterized tumor associated markers. Its increased plasma levels and cellular expression associated with inflammatory diseases, smoking and various solid tumor malignancies have puzzled researchers for many years. Immunological and physicochemical characterization of CEA has revealed both an inherent microheterogeneity and an association with other glycoproteins. These facts have stimulated research into the biological function(s) of CEA and knowledge of the genetics of CEA would help in clarifying the relationship of the CEA "family" of antigens and might help in defining its biological function(s). One approach to mapping the CEA gene(s) is somatic cell hybridization. The establishment of long term colonic tumor cell lines which produce CEA has provided a means of studying CEA in vitro. The development of specific monoclonal antibodies to CEA and to the CEA related glycoproteins has provided a tool for analysis of antigen expression by hybrid cells. Once the CEA gene(s) can be located, further genetic investigation should make it possible to study the expression of these genes in normal and malignant tissues.

1.4 Statement of objectives

The objectives of this project were:

1. To produce, by somatic cell hybridization, interspecific human-mouse hybrids between colorectal, CEA expressing/secreting, cancer cell lines and mouse cell lines.
2. To screen the resultant hybrids for CEA expression/secretion using specific immunological assays (enzyme linked immunosorbent assay, immunoperoxidase assay).
3. To characterize hybrids for human chromosome content using chromosomal analysis techniques (Giemsa differential staining, Giemsa banding).
4. If objectives 1-3 were successfully attained and CEA expressing-secreting hybrids were defined, then an attempt would be made to map the CEA gene(s) by correlating antigen expression with the presence of specific chromosome(s) in the hybrid cells.

Chapter 2

MATERIALS AND METHODS

2.1 Cell Lines

The human cell lines selected as fusion partners for the production of interspecific hybrids were SKCO1 and LS174T (high CEA expressors), and HT29 (low to moderate CEA expressor).

2.1.1 LS174T

This cell line was derived as a trypsinized variant from the primary culture LS180. LS180 was established in 1974 from a moderately well differentiated primary colonic adenocarcinoma removed from a 58 year old woman (Tom, Rutzky, Jakstys, Oyasu, Kaye and Kahan, 1976). LS174T, a monolayer culture cell line, has been characterized for CEA expression and production, morphology, karyology and growth properties (Tom et al., 1976; Kahan, Rutzky, Berlin, Tomita, Wiseman, Legrue, Noll and Tom, 1976; Rutzky, Kaye, Siciliano, Chao and Kahan, 1980; Sheer, Brown and Bobrow, 1982; Shi et.al., 1983; Ford et al., 1987).

2.1.2 SKCO1

This cell line was established in 1972 from a malignant ascites from a 65 year old male with colonic adenocarcinoma (Fogh and Trempe, 1975). SKCO1, a monolayer culture cell line, has been characterized for CEA expression and production (Shi et al., 1983; Ford et al., 1987).

2.1.3 HT29

This cell line was established in 1964 from a moderately, well differentiated, grade II, colonic adenocarcinoma from a 44 year old female (Fogh and Trempe, 1975). HT29, a monolayer culture cell line, has been characterized for CEA expression and production (Egan and Todd, 1972; Shi et al., 1983; Ford et al., 1987).

All three had previously been characterized for CEA expression in this laboratory using enzyme-linked immunosorbent assay (ELISA), immunoperoxidase and in vitro antibody binding assays with monoclonal anti-CEA antibodies (Ford, Bartlett, Casson, Marsden and Gallant, 1987; Table 8).

Table 8. CEA Characteristics of human colorectal adenocarcinoma cell lines.

Cell Lines	Immunocytochemistry+	No. antibodies binding per cell ($\times 10^4$)++	CEA production (ng CEA per 10^6 cells)*
1. LS174T	60	48	86
2. SKC01	82	110	48
3. HT29	4	3	5

+ % of cells CEA positive
++ Data obtained using ^{125}I labelled monoclonal anti-CEA (11-285-14)
* Data obtained from Shi, Tsao and Kim, 1983.

The mouse cell lines chosen as fusion partners for the production of interspecific hybrids were NS-1 and SP2/0 (mouse myelomas); RAG (renal adenocarcinoma); PG19 (melanoma) and STO (embryonic fibroblast). The recommended growth media are indicated in Table 9 (The Human Genetic

Mutant Cell Repository, 1981; American Type Culture Collection, 1983).

Table 9. Recommended culture media plus growth supplements.

Cell Line	Culture Medium	Supplements
1.LS174T SKCO1	Eagle's Minimum Essential medium (500ml)	50 ml FCS 6 ml Glutamine 12 ml Penicillin/Streptomycin 6 ml Nonessential amino acids
2.HT29	McCoy's Iwakata and Grace medium (500ml)	100 ml FCS 6 ml Glutamine 12 ml Penicillin/Streptomycin 6 ml Nonessential amino acids 15 ml HEPES buffer
3.NS-1 SP2/0	RPMI 1640 medium (500ml)	50 ml FCS 6 ml Glutamine 12 ml Penicillin/Streptomycin 10 ml Glucose (4.5g per liter) 1250 ul 6-Thioguanine (10^{-2}M)
4.RAG	Eagle's Minimum Essential medium (500ml)	50 ml FCS 6 ml Glutamine 12 ml Penicillin/Streptomycin 6 ml Non essential amino acids 6 ml Vitamins 1250 ul 6-Thioguanine (10^{-2}M)
5.PG19	Dulbecco's Modification of Eagle's medium (500ml)	50 ml FCS 6 ml Glutamine 12 ml Penicillin/Streptomycin 1250 ul 6-Thioguanine (10^{-2}M)
6.STO	Dulbecco's Modification of Eagle's medium (500ml)	50 ml FCS 6 ml Glutamine 12 ml Penicillin/Streptomycin 10 ml Glucose (4.5g per liter) 1250 ul 6-Thioguanine (10^{-2}M)

2.2 General Tissue Culture Techniques

All tissue culture procedures were carried out aseptically in laminar flow containment cabinets (Level II).

2.2.1 Trypsinization

A. Materials

(1) Trypsin-EDTA (10x, Gibco Laboratories)

(2) 0.15M Phosphate Buffered Saline (PBS) pH 7.2

B. Method

(1) A 1:9 trypsin and PBS solution was prepared.

(2) Each monolayer culture was washed twice with PBS and then incubated with the diluted trypsin solution for 5-10 minutes at 37°C.

(3) Once the cells had lifted off the plastic, the cell suspension was centrifuged at 200 g for 5 minutes in a benchtop centrifuge (IEC HN SII model). Following this the supernatant was decanted, the cell pellet was resuspended in the recommended growth medium (Table 9) and a viability cell count was performed (Section 2.2.4).

2.2.2 Freezing Cells

A. Materials

(1) Dimethyl sulfoxide (DMSO, BDH Chemicals)

(2) Fetal Calf Serum (FCS, Gibco Laboratories)

(3) Freezing vials (Nunc)

B. Methods

(1) Cell cultures were trypsinized, assessed by a viability cell count and washed twice with PBS.

(2) Following washing, cells were centrifuged at 200 g for 5 minutes in the benchtop centrifuge (IEC HN SII model) and then resuspended in approximately 1 ml of cold FCS (90%)/DMSO (10%) per 4-6x10^6 cells. The 1 ml aliquots were then quickly transferred to freezing vials, stored in -70°C for 2-3 days and then transferred to liquid nitrogen (container type, APOLLO SX 35, MVE).

2.2.3 Thawing Cells

A. Materials

(1) Recommended growth medium (see Table 9)

(2) Culture flasks (75 cm^2 or 25 cm^2)

(3) 15 ml centrifuge tubes

B. Method

(1) The freezing vial was removed from liquid nitrogen storage, quickly thawed in a 37°C waterbath until a small ice pellet remained and then placed on ice.

(2) The thawed cell solution was quickly diluted 1:14 in recommended growth medium and then centrifuged at 200 g for 5 minutes.

(3) Following centrifugation, the supernatant was decanted and the pellet was resuspended in 5 ml of recommended growth medium. Then the cells were assessed for viability (see Section 2.2.4), put into culture flasks and incubated in a 37°C humidified incubator with 5% CO_2.

(4) After 24 hours the cells were supplemented with an additional 5-10 ml of growth medium.

2.2.4 Viability Test (Acridine Orange/Ethidium Bromide)

A. Materials

(1) Hemacytometer counting chamber

(2) Acridine orange/ethidium bromide Stain (AO/EB) (Sigma Chemical Co.)

The solution was prepared by dissolving 0.1 mg of both AO and EB in 100 ml of PBS. This was divided into aliquots and frozen at -20°C.

(3) Light microscope, ultraviolet light source (Leitz Ortholux II)

B. Method

(1) After recording the total volume of the cell suspension, one drop was aseptically removed and added to one drop of AO/EB solution.

(2) Following this, the mixed solution was added to the hemacytometer and the cell viability assessed under ultraviolet light.

(3) First, the total acridine orange stained viable cells (fluorescent green) and the ethidium bromide stained dead cells (brown) are counted. Then the viability was calculated with the following equation:

% viability = (Total AO cells/ Total cell count) x 100%

2.3 Human-Mouse Interspecific Fusions

2.3.1 General Materials

A. Fusing agents

(1) Polyethylene Glycol 1500 (BDH Chemicals)

(2) Polyethylene Glycol 4000 (J.T. Baker)

Polyethylene glycol (PEG)	25 g
RPMI 1640	22.5 ml
DMSO	2.5 ml

First, the RPMI 1640 and DMSO solutions were mixed and the PEG was melted. Following this, the PEG was mixed with the RPMI 1640/DMSO mixture and sterilized by autoclaving. Finally, 2 ml aliquots of the sterile fusing solution were dispensed into sterile 15 ml tubes and stored at -20°C. This preparation was 50% PEG and 5% DMSO.

B. Chemical Selection Solutions

(1) Hypoxanthine-Thymidine (100x) and (50x) stock solutions

Hypoxanthine	0.0307 g	(Sigma Chemical Co.)
Thymidine	0.1361 g	(Sigma Chemical Co.)

The two preformed bases, hypoxanthine and thymidine, were dissolved in approximately 75 ml of 60-70°C prewarmed distilled water. Following this, the total volume was adjusted to 100 ml, sterilized by filtering through a 0.22 um filter and stored at -20°C. This is a 100x HT solution. The 50x HT solution was prepared by adding an equal volume of distilled water to the 100x HT

solution. Again, the solution was sterilized and stored in a similar manner.

(2) HT selective medium

HT (50x)	2 ml
RPMI 1640-FCS-glutamine	98 ml

The final concentration for hypoxanthine and thymidine was 1.0×10^{-4} M and 1.6×10^{-5} M respectively.

(3) Hypoxanthine-Aminopterin-Thymidine (50x) stock solution

Aminopterin 0.0176 g (Sigma Chemical Co.)

The aminopterin was dissolved with a few drops of 0.1 M NaOH in approximately 80 ml of distilled water and then the final volume was adjusted to 100 ml. The HAT (50x) solution was prepared by mixing 10 ml of the aminopterin solution with 100 ml of HT (100x) solution and 90 ml of distilled water. Following this, the solution was sterilized by filtering through a 0.22 um filter and stored at -20°C.

(4) HAT selective medium

HAT (50x)	2 ml
RPMI 1640-FCS-glutamine	98 ml

The final concentrations for hypoxanthine, aminopterin, and thymidine were 1.0×10^{-4} M, 4.0×10^{-7} M and 1.6×10^{-5} M respectively.

(5) Ouabain stock solution

Ouabain 0.0365 g (Sigma Chemical Co.)

Ouabain was dissolved in 5 ml of distilled water, sterilized by filtering through a 0.22 um filter and stored at 4°C. The concentration of the ouabain stock solution was 1.0×10^{-2} M.

(6) Ouabain selection medium

Ouabain stock solution 100 ul

RPMI 1640-HAT-FCS-glutamine 100 ml

The final ouabain concentration was 1.0×10^{-5} M.

(7) 6-Thioguanine stock solution

6-Thioguanine 0.167 g (Sigma Chemical Co.)

Distilled water 100 ml

The 6-thioguanine (6-TG) was dissolved with a few drops of 10 M NaOH in approximately 90 ml of distilled water. The solution was then adjusted to a final volume of 100 ml with distilled water, sterilized by filtering through a 0.22 µm filter and stored at -20°C. The final concentration of 6-TG was 1.0×10^{-2} M.

(8) 6-Thioguanine selection medium for mouse HGPRT negative cells

6-TG stock solution	1.25 ml
Recommended growth medium	600 ml

The final 6-TG concentration was 2×10^{-5} M.

Note: The standard culture media bottles contain 500 ml, however, after addition of supplements the volume totals approximately 600 ml.

2.3.2 Fusion Methods

A. Standard "Suspension" Fusion Method

The standard method for producing human-mouse hybrids was a modification of the suspension fusion technique used for the production of mouse-mouse hybridomas (Kohler and Milstein, 1975). The method followed these basic steps:

(1) A viability cell count was performed on both fusion partners. The lowest percentage viability accepted for both cell lines was 85%.

(2) If the cell viability was above 85% for both cell lines the cells were washed in PBS twice and mixed at a 5:1 human to mouse cell ratio in a 50 ml centrifuge tube. Following this, the cell mixture was centrifuged at 500 g in a benchtop centrifuge (IEC HN SII model) for 5 minutes.

(3) After the cells were pelleted, the supernatant was decanted and

the cells were gently resuspended. For addition of the fusing agent, PEG/DMSO, the temperature was maintained at 37°C.

(4) One ml of PEG/DMSO/RPMI 1640 was added slowly over 1 minute followed by an additional minute of stirring.

(5) Ten ml of RPMI 1640 was added slowly to the PEG/DMSO fusion mixture over 5 minutes. Following this, the diluted mixture was centrifuged at 200 g in the benchtop centrifuge for 5 minutes.

(6) After the centrifugation the supernatant was decanted, the pellet was resuspended in 25 ml of RPMI-HAT-Ouabain-FCS and the resultant cell solution was dispensed in 50 ul aliquots per well in 96 well culture plates which already contained 100 ul of selection medium.

(7) Finally, the culture plates were incubated in a gassed 5% CO_2 37°C controlled environment incubator and the medium was replaced twice weekly using the following selection schedule:

2 weeks in HAT-Ouabain selection

1 week in HT-Ouabain selection

1 week in normal medium

B. Modifications to the Standard Fusion Method

The initial set of fusions followed the standard method. The failure to produce human-mouse hybrids prompted the evaluation of the following modifications to the standard method including:

(1) different fusion cell ratios including 1:1, 10:1, and 15:1,

(2) different molecular weight PEG 4000,

(3) different plating densities,

(4) different mouse cell lines including an additional suspension cell line and three monolayer cell lines.

Illustrated in Table 10 are the different fusion protocols with the modifications evaluated.

Table 10. Different fusion protocols.

Protocol	PEG	Fusion Partners Human	Mouse	Ratio Human/mouse	Plating Density	No. of Fusions
1	1500	LS174T HT29 SKCO1	NS1	5:1	2.5×10^4 to 6.4×10^4	10
2	1500	LS174T HT29 SKCO1	NS1 SP2/O	10:1,15:1	3.4×10^4 to 6.6×10^4	9
3	1500	HT29 SKCO1	NS1 SP2/O	1:1,5:1 and 10:1	1.25×10^5	12
4	4000	HT29 SKCO1	NS1 SP2/O	1:1,5:1 and 10:1	2.5×10^4	12
5	4000	HT29 SKCO1	RAG STO PG19	1:1	2.5×10^4	5
					Total Fusions = 48	

2.4 Cloning by Limiting Dilution

This method was based on the one described by Hudson and Hay (Hudson and Hay, 1980). The rationale for cloning was to enrich for CEA producing hybrids.

A. Materials

(1) Hybrid cells

(2) 96 and 24 well flat bottom culture plates (Linbro)

B. Method

(1) The hybrid cells were grown in 25 cm^2 tissue culture flasks prior to the cloning process.

(2) The cells were trypsinized, viability was assessed and cell dilutions were prepared at 10 and 5 cells per ml for each hybrid.

(3) For each hybrid one 96 well culture plate was set up. One half of the plate received 50 ul per well of the 10 cell per ml dilution and the other half received 50 ul per well of the 5 cell per ml dilution. Assuming the dilutions were correct then one half of the plate received 25 cells per 48 wells and the other half received 12.5 cells per 48 wells.

(4) Each culture plate was incubated in the 5% CO_2 37°C incubator. After 7-10 days the plates were screened with the inverted phase microscope for the presence of hybrid clones. Only the wells containing single monolayer colonies were accepted as true clones. All culture plates set up for cloning were screened for 4 weeks before discarding.

(5) Those wells containing single monolayer colonies were expanded to 24 well culture plates. When they reached confluence spent medium samples were collected and tested in the ELISA (see Section 2.8.1).

(6) Finally, all clones were frozen for long term storage (see Section 2.2.2). The initial distribution of cells per well follows Poisson statistics, thus although about 60% of the wells will receive only one cell and therefore initiate a true clone, a significant proportion will receive 2 or more cells. Cloning must be repeated to ensure the homogeneity of any interesting hybrid line (Hudson and Hay, 1980).

2.5 Solubilization of CEA from Cells

The following method was used to obtain membrane and cytosol fractions from various cells (parent and hybrid) for CEA determinations by ELISA (Shi et al., 1983).

A. Materials

(1) TNEN buffer [Trizma base (Tris hydroxylmethyl aminomethane), NaCl, EDTA (ethylenediamine tetraacetate acid disodium salt), NP40 (Nonidet P-40)]

This buffer was prepared by dissolving the following amounts of each component in approximately 80 ml distilled water.

Trizma base	0.242 g	(Sigma Chemicals)
NaCl	0.585 g	(BDH Chemicals)
EDTA	0.037 g	(Sigma Chemical Co.)
NP40	500 ul	(Sigma Chemical Co.)

After dissolving the components the pH was adjusted to 8.0 and the final volume made up to 100 ml with distilled water.

(2) Beckman ultracentrifuge (Model L5-65)

(3) Beckman centrifuge rotor (Type 75 Titanium Fixed Angle)

B. Method

(1) Cells were grown in 8x75 cm^2 tissue culture flasks. When the cells were confluent they were trypsinized, washed twice with phosphate buffered saline (pH 7.4), counted and frozen at -70°C. Samples of spent medium from each cell type were removed for CEA analysis by ELISA.

(2) Following this, the cells were thawed at 4°C, sonicated twice for 15 seconds and divided into two volumes (2/3,part A;1/3,part B).

(3) Refer to Figure 1 for the rest of the procedure.

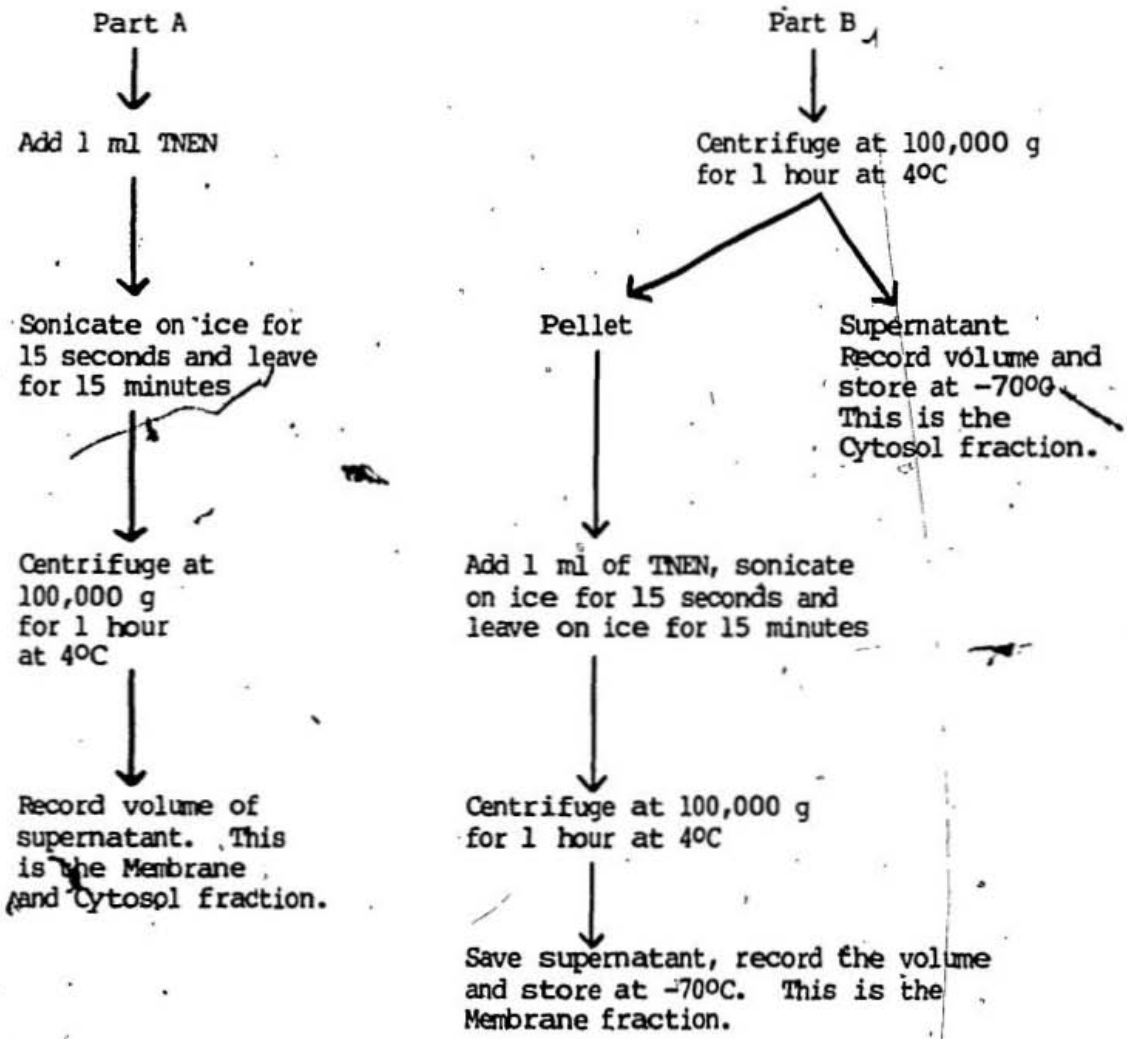

Figure 1. Outline of solubilization method.

2.6 Concentration of Spent Medium

A. Materials

(1) Minicon concentrators (Eight-cell capacity, 5 ml each, 15,000 molecular weight cutoff; Amicon Canada Ltd.).

(2) Spent medium samples collected from confluent monolayer cultures (75 cm^2).

B. Method

(1) Spent medium samples from selected hybrid cultures were concentrated five-fold in the minicon concentrators.

(2) After collecting the concentrated spent medium samples, they were assayed for CEA by the ELISA (Materials and Methods, section 2.8.1).

2.7 Chromosomal Analysis

2.7.1 Chromosome Harvesting

A. Materials

(1) Colcemid (Gibco Laboratories)

(2) KCl (Fisher Scientific Co.)

(3) Acetic acid, glacial (Fisher Scientific Co.)

(4) Methanol (Fisher Scientific Co., A412-4)

B. Method

(1) Cell cultures were set up in 25 cm^2 tissue culture flasks and incubated at 37°C.

(2) The cultures were observed daily with the inverted phase light microscope to determine the optimal time for chromosome harvesting. This was indicated by the presence of mitotic cells. Harvesting was started when the number of mitotic cells exceeded twenty-five.

(3) Once it was decided to harvest, cultures were incubated in recommended growth medium plus colcemid at a final concentration of 0.05 ug per ml for 15-20 minutes. Following this, the medium was poured into labelled 15 ml centrifuge and the cells were trypsinized (see Section 2.2.1). As soon as the cells detached, the cell suspension was poured into the appropriately labelled centrifuge tubes.

(4) The cells were centrifuged at 200 g for 5 minutes in a benchtop centrifuge (IEC HN SII model). After centrifugation the supernatant was decanted, the cells were gently resuspended and approximately 5-10 ml of prewarmed 37°C KCl (0.075 M) was added. Then, the cells were incubated approximately 10-15 minutes in a 37°C waterbath.

(5) After hypotonic treatment, the cells were centrifuged at 200 g for 5 minutes in the benchtop centrifuge. Following centrifugation, the supernatant was decanted, the cell pellet was gently resuspended and freshly prepared acetic acid/methanol (1:3) was added to the cells. Fixative (acetic acid-methanol) was added dropwise for the initial 1-2 ml to avoid cell clumping.

(6) After the addition of fixative, the cells were centrifuged at 200 g for 5 minutes in the benchtop centrifuge. Once the fixative

was changed three times, chromosome spreads were prepared.

(7) Chromosome spreads were prepared by dropping approximately 50 to 100 ul of cell suspension on to ethanol precleaned slides held at a 45° angle. Then, the slides were heat dried at 95°C for 30 minutes.

2.7.2. Chromosome Staining

2.7.2.1. Giemsa Banding

The Giemsa banding method, developed by Wang and Fedoroff, was used to band human and mouse chromosomes (Wang and Fedoroff, 1972).

A. Materials

(1) Wright's Stain (Sigma Chemical Co.)

(2) 0.05% Bactotrypsin (Difco Laboratories)

(3) Saline (BDH Chemicals)

(4) Phosphate buffer pH 7.0 (Sorensen's)

B. Method-Part 1: Preparing Solutions

(1) 0.05% Bactotrypsin in 0.9% saline (NaCl)

The lyophilized vial of bactotrypsin was rehydrated with 20 ml of PBS pH 7.4 to give a 2.5% trypsin solution. After rehydration the solution was aliquotted in 1 ml amounts into plastic 1.5 ml Eppendorf tubes and stored at -20°C. The 0.05% trypsin solution was prepared by mixing 1 ml 2.5% trypsin with 49 ml of 0.9% saline.

(2) Sorensen's stock buffer, pH 7.0

KH_2PO_4 4.536 g (J.T. Baker Chemical Co.)

Na_2HPO_4 4.733 g (BDH Chemicals)

Each chemical was dissolved separately in 500 ml of distilled water. The Sorensen's stock buffer was prepared by mixing 61.1 ml of Na_2HPO_4 with 38.9 ml of KH_2PO_4.

(3) Phosphate buffer, pH 6.8 (5% working solution)

The 5% phosphate buffer was prepared by diluting 5 ml of the Sorensen's stock buffer with 95 ml of distilled water.

(4) Wright's stock stain

Wright's 1.0 g (Sigma Chemical Co.)

The Wright's stock stain was prepared by dissolving 1 gram in 250 ml of methanol. This was stirred for 2 hours at room temperature and then filtered to remove any undissolved powder (Whatman's #1 filter paper).

(5) Wright's stain (working solution)

This was prepared by mixing 1 ml of Wright's stock stain with 3 ml of 5% phosphate buffer, pH 6.8.

B. Method-Part 2: Banding Chromosomes

(1) The slide preparation was dipped in the 0.05% trypsin solution for 10-60 seconds. Immediately following this, the slide was rinsed in 0.9% saline twice, and stained with Wright's working solution for 10-60 seconds.

(2) After the staining period the stain was washed off with tap water, blotted partially dry with gauze and fully dried with a hot air dryer.

(3) With oil immersion light microscopy the Giemsa banding was assessed. If the chromosomes were not banded enough a second slide was exposed in trypsin for a longer time. If the chromosomes were too swollen then the trypsin time was decreased.

(4) Well banded metaphases were photographed (Section 2.7.3.1).

2.7.2.2. Differential Giemsa Alkaline Staining

The following is a modification of the differential staining technique described (Alhadeff, Velivasakis and Siniscalco, 1977).

A. Materials

(1) Giemsa alkaline stock solution

(2) 0.05 M Phosphate buffer, pH 11.3

B. Method-Part 1: Preparing Solutions

(1) Giemsa-11 stock

Giemsa stain powder	1.0 g	(Fisher Scientific, G-146)
Glycerol	66 ml	(Sigma Chemical Co.)
Absolute ethanol	66 ml	

One gram of Giemsa stain powder was mixed with 6 ml of pre-warmed 60°C glycerol in a 250 ml Erlenmeyer flask. This mixture was grounded with a round-bottom glass test tube and diluted with an additional 60 ml of prewarmed glycerol. Following this, the flask was wrapped in aluminum foil, stirred for 1 hour at room

temperature, transferred to a shaker waterbath at 60°C and shaken overnight. The next day the solution was removed from the waterbath, cooled, supplemented with 66 ml of absolute ethanol and stirred for 2-3 hours at room temperature.

(2) 0.05M sodium phosphate buffer pH 11.3

Na_2HPO_4	3.549 g	(Fisher Scientific Co.)
$NaH_2PO_4H_2O$	3.450 g	(Fisher Scientific Co.)

Both portions were dissolved in approximately 800 ml of distilled water, the pH was adjusted to 11.3 with 10 M NaOH and the final volume was adjusted to 1000 ml with distilled water.

B. Method-Part 2: Differential Staining

(1) Slides, aged for 2-3 days, were preincubated for 2 hours at 60°C in distilled water. Twenty minutes before the 2 hour incubation was completed the 6% Giemsa working solution was prepared. Three ml of Giemsa stock solution was diluted with 47 ml of prewarmed 37°C 0.05 M phosphate buffer, pH 11.3.

(2) The stain was then centrifuged at 500 g for 5 minutes in a benchtop centrifuge (IEC HN SII model) to remove nondissolved powder. After centrifugation the stain was carefully decanted into a Coplin staining jar and equilibrated to 37°C in a waterbath.

(3) Once the 2 hour incubation was completed the slides were removed, air dried and placed into the 6% Giemsa working solution for a time period ranging from 2-10 minutes. A film that forms on top of the staining solution was carefully wiped off with folded absorbent tissues before the slides were dipped in and before they

were removed.

(4) The slides are assessed for good quality differentially stained metaphases with oil immersion light microscopy.

(5) Well stained metaphases were photographed (Section 2.7.3.2).

2.7.3 Photomicroscopy

2.7.3.i Black and White (Giemsa Banding)

A. Materials

(1) The equipment required for photographing Giemsa banded chromosomes included a light microscope (Wild Leitz ortholux II) equipped with an interchangeable 35 mm camera and Photoautomat MPS 45 (Wild Leitz).

(2) The materials required for developing the 35 mm technical pan film included film developer solution (Kodak Developer-76) and film fixer solution (Kodak Rapid Fixer).

(3) The materials and equipment required for printing the 35 mm film included printing paper (Kodak Ektamatic SC paper), activator solution (Kodak SII activator), stabilizer solution (Kodak Ektamatic S30 stabilizer), enlarger and automatic printer.

B. Method-Part 1: Photography

(1) All Giemsa banded metaphases were photographed under oil immersion with the 35 mm interchangeable camera photoautomat MPS 45 system (100x objective).

B. Method-Part 2: Development

(1) In a darkroom the film was loaded into the developing cassette.

(2) The film was developed for 6-10 minutes in 1:1 developing solution and tap water (Developer-76/water) at 20-22°C. The cassette was gently inverted every 30 seconds for uniform film developing.

(3) After the developing time the solution was decanted, the film was thoroughly rinsed with tap water at 20-22°C for 30-60 seconds and the film fixed for 2-4 minutes in fixing solution. Again, the cassette was gently inverted every 30 seconds for uniform film fixing.

(4) After the fixing time period the solution was decanted, the film was thoroughly washed in tap water and dried with a hot air dryer.

B. Method-Part 3: Printing

(1) All printing was performed with the film enlarger and automatic printer in a darkroom.

(2) The automatic printer was filled with both activator and stabilizer solutions.

(3) The 35 mm film was placed in the enlarger film feeder and each negative was focussed and exposed to Ektamatic SC paper for 1-15 seconds.

(4) Immediately following this the paper was processed by the automatic printer and air dried.

2.7.3.2 Color

The equipment required for photographing Giemsa differentially stained chromosomes was the same as that used for Giemsa banding. All color slides were processed by the medical audio-visual unit of the Faculty of Medicine, Memorial University of Newfoundland.

2.8 CEA Screening Techniques

2.8.1. Enzyme Linked Immunosorbent Assay (ELISA)

This is a modification of the method used to measure anti-CEA antibodies (Woodhouse, Ford, and Newman, 1982; Ford et al., 1987) and that used for measuring CEA (Casson, Ford, Marsden, Gallant and Bartlett, 1987).

A. Materials

(1) Rabbit anti-CEA immunoglobulin (DAKO, Denmark)

(2) Monoclonal mouse anti-CEA antibody (11-285-14)

(3) Peroxidase conjugated goat-anti rabbit immunoglobulins (Miles Scientific Inc.)

(4) Affinity purified CEA standards ranging in concentration from 3 ng per ml to 100 ng per ml in PBS/BSA diluent.

(5) Substrate reagent - ABTS 27.8 ng per ml (Sigma Chemical Co.)
ABTS - [2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid)]

(6) Saline-Tween (washing solution)

0.15M NaCl

0.1% Tween 20 (BDH Chemicals)

(7) Carbonate-bicarbonate buffer, (0.1M pH 9.2)

Na_2CO_3 0.795 g

$NaHCO_3$ 1.465 g

The carbonate and bicarbonate were each dissolved separately in approximately 400 ml of distilled water, adjusted to pH 9.2 with 6M HCl and the final volume made up to 500 ml with distilled water.

(8) 1% BSA-Carbonate buffer (pH 9.2, 0.1M)

Bovine serum albumin (BSA) 1 g (Sigma Chemicals)

Carbonate-bicarbonate buffer 100 ml

(9) Citrate phosphate buffer, 0.1M pH 4.0

Citric acid 4.53 g (BDH Chemicals)

Na_2HPO_4 4.53 g (Fisher Scientific)

The two components were dissolved in approximately 400 ml of distilled water, adjusted to pH 4.0 with 1 M HCl and the final volume adjusted to 500 ml with distilled water.

(10) 1% BSA-PBS-Tween Diluent

BSA 1 g

PBS pH 7.2 100 ml

Tween 20 100 ul

B. Method-Part 1: Coating Cuvettes

(1) The coating solution was prepared by making the appropriate dilution of affinity purified 11-285-14 antibody with 0.1 M carbonate-bicarbonate buffer (pH 9.2) to give a 2.5 ug per ml solution.

(2) The coating solution was dispensed, 250 ul per cuvette, with the EIA "50" ELISA system (Gilford).

(3) The cuvettes were sealed, incubated for 3 hours in a 37°C waterbath and stored at 4°C until the assay was performed.

B. Method-Part 2: The CEA Assay

(1) Coating solution was removed and cuvettes automatically washed 6x with saline-Tween. The 1% BSA-carbonate buffer pH 9.2 was dispensed, 300 ul per well, with the EIA "50" ELISA system. The cuvettes were sealed and incubated for 1 hour in a 37°C waterbath.

(2) 200 ul of the appropriate controls (1%BSA/PBS; HAT-RPMI; CEA standards) and test samples were dispensed into separate cuvette wells with a Gilson micropipette, the cuvettes were sealed and incubated for 2 hours in a 37°C waterbath. After each incubation stage the cuvettes were automatically washed 6x with saline-Tween washing solution to remove any nonbound material.

(3) A 1:2000 dilution of the second antibody (Dako rabbit anti-CEA) with diluent (1% BSA-PBS-Tween) was prepared, dispensed (250 ul per well) and incubated for 2 hours in a 37°C waterbath.

(4) A 1:4000 dilution of the conjugate (goat anti-rabbit horse radish peroxidase) with diluent was prepared, dispensed (250 ul per well) and incubated for 2 hours in a 37°C waterbath.

(5) The substrate reaction mixture was prepared by mixing together 100 ul of stock ABTS (27.8 mg per ml), 1 ul of H_2O_2 (30%) and 12.5 ml of citrate phosphate buffer (pH 4.0)

(6) The reaction mixture was dispensed, 250 ul per well, with the EIA "50" ELISA system. The absorbance readings at 405 nm were measured for time periods ranging from 0-60 minutes. The absorbance for each cuvette well at T=0 minutes were used as background levels and were subtracted from the final readings.

2.8.2 Immunoperoxidase Assay

The following two stage indirect immunoperoxidase assay, developed by Heyderman (1979) and modified by Ford, Gallant and Ali (1985), was used to investigate CEA expression in the interspecific hybrids.

A. Materials

(1) The antibodies used in this assay include a mouse anti-CEA monoclonal antibody (11-285-14), a rabbit anti-CEA polyclonal antibody (Dako, Denmark), nonspecific mouse antibody (control ascites, Bethesda Research Laboratories), normal rabbit serum, normal sheep serum, rabbit anti-mouse horse radish peroxidase conjugate (RAM-HRP)(DAKO) and goat anti-rabbit horse radish peroxidase conjugate (GAR-HRP)(Miles Scientific).

(2) The solutions used in this assay include the following:

Xylene

Ethanol (Absolute, 75%, 60%, 30%)

Phosphate buffered saline, pH 7.4

Hydrogen Peroxide, 7.5% (H_2O_2 30%/stock, Anachemia Ltd.)

Periodic acid, 2.28% (periodic acid, 95% stock, BDH Chemicals)

Potassium borohydride, 0.02% (BDH Chemicals)

BSA in PBS, 1.0%

Brij 35 in PBS, 0.001% (Brij 35, 30% stock, Technicon)

Mayer's haemalum stain (Harleco Diagnostics, BDH)

Lithium carbonate, saturated aqueous solution

Permount (Fisher Scientific)

(3) The substrate reaction mixture consisted of the following

3',3'-Diaminobenzidine	30 mg	(Sigma Chemicals)
30% H_2O_2	60 ul	
PBS, pH 7.4	60 ml	

(4) The slides tested in each assay included methanol-fixed smears of human and mouse parent cells, methanol-fixed smears of hybrid cells and formalin-fixed, paraffin-embedded tissue sections from colorectal carcinoma liver metastases.

B. Method

All procedures were carried out at room temperature.

(1) The following solutions were prepared before starting the assay: 7.5% H_2O_2; 2.28% Periodic acid; 0.02% Potassium Borohydride; 1.0% BSA in PBS and 0.001% Brij 35 in PBS.

(2) The slides were bleached with 7.5% H_2O_2 for 5 minutes and, subsequently, rinsed thoroughly with tap water to remove the hydrogen peroxide.

(3) The slides were then incubated in 2.28% periodic acid for 5 minutes and, subsequently, rinsed thoroughly with tap water.

(4) To block all free aldehyde groups the slides were incubated in 0.02% potassium borohydride for 2 minutes. Subsequently the slides were rinsed with tap water and then with PBS.

(5) To block nonspecific binding of the conjugate a 10 minute incubation in a humidified chamber was performed using 50 ul per slide of the following blocking agents:

1:25 normal rabbit serum if 11-285-14 and RAM-HRP were used

1:25 normal sheep serum if DAKO anti-CEA and GAR-HRP were used

(6) The slides were washed in fresh 0.001% Brij 35 in PBS for 5 minutes.

(7) After the washing stage, the slides were incubated in a humidified chamber for 30 minutes with 50 ul per slide of one of the following test and control antibodies:

11-285-14 (test,10 ug ml^{-1}), control ascites (control,10 ug ml^{-1})

DAKO anti-CEA (test,1:1000), normal rabbit serum (control,1:1000)

(8) The slides were washed in fresh 0.001% Brij 35 in PBS for 15 minutes.

(9) After the washing stage, the slides were incubated in a humidified chamber at room temperature for 30 minutes with 50 ul of one of the following conjugates:

RAM-HRP (1:50) if 11-285-14 was used

GAR-HRP (1:50) if DAKO anti-CEA was used

(10) Step #8 was repeated.

(11) The slides were incubated in freshly prepared substrate solution for 5 minutes and subsequently rinsed with PBS.

(12) Following this, the slides were stained in Mayer's haemalum stain for 5 minutes and subsequently rinsed with tap water.

(13) After staining the slides were soaked in lithium carbonate for 30-60 seconds and then dehydrated by rinsing through the alcohols. Finally the slides were rinsed in xylene and mounted in Permount.

(14) All slides were screened for membrane and cytosol CEA expression with the light microscope (25x objective).

Chapter 3

RESULTS

3.1 Giemsa Banding Parent Cell Lines

Representative karyotypes were determined using the Giemsa chromosomal banding technique. The reasons for obtaining karyotypes of each cell line were to identify any marker chromosomes that might be part of any human-mouse hybrids produced in this project and to become familiar with chromosome analysis techniques. The basic chromosomal harvesting method used for obtaining and preparing chromosome spreads was the conventional "colcemid-hypotonic-fixative" technique (Seabright, 1971). Chromosomal ranges, determined by analyzing approximately 100 metaphases for each cell line, are shown in Table 11.

Table 11. Chromosome ranges for LS174T, SKCO1 and HT29.

Human Cell Line	Chromosomal Range (% metaphases within this range)
LS174T	43-47 (81.8)
HT29	67-74 (83.6)
SKCO1	70-77 (84.0)

Giemsa banding of the cell lines proved to be difficult and problems encountered included low mitotic indices, poor quality chromosome spreads and poor banding. Attempts were made to improve the number of metaphases and the quality of the chromosome spreads with cell synchronization methods including aminopterin-thymidine and actinomycin D synchronization

(Yunis, 1981; Wiley, Sargent, Inhorn and Meisner, 1984). However, no improvement was observed and the synchronization experiments were discontinued. Although problems were encountered with chromosomal analysis of the human cell lines representative cells were karyotyped. Karyotypes of HT29 and LS174T are illustrated in Plates 1 and 2 and an SKCO1 Giemsa banded metaphase cell is depicted in plate 3.

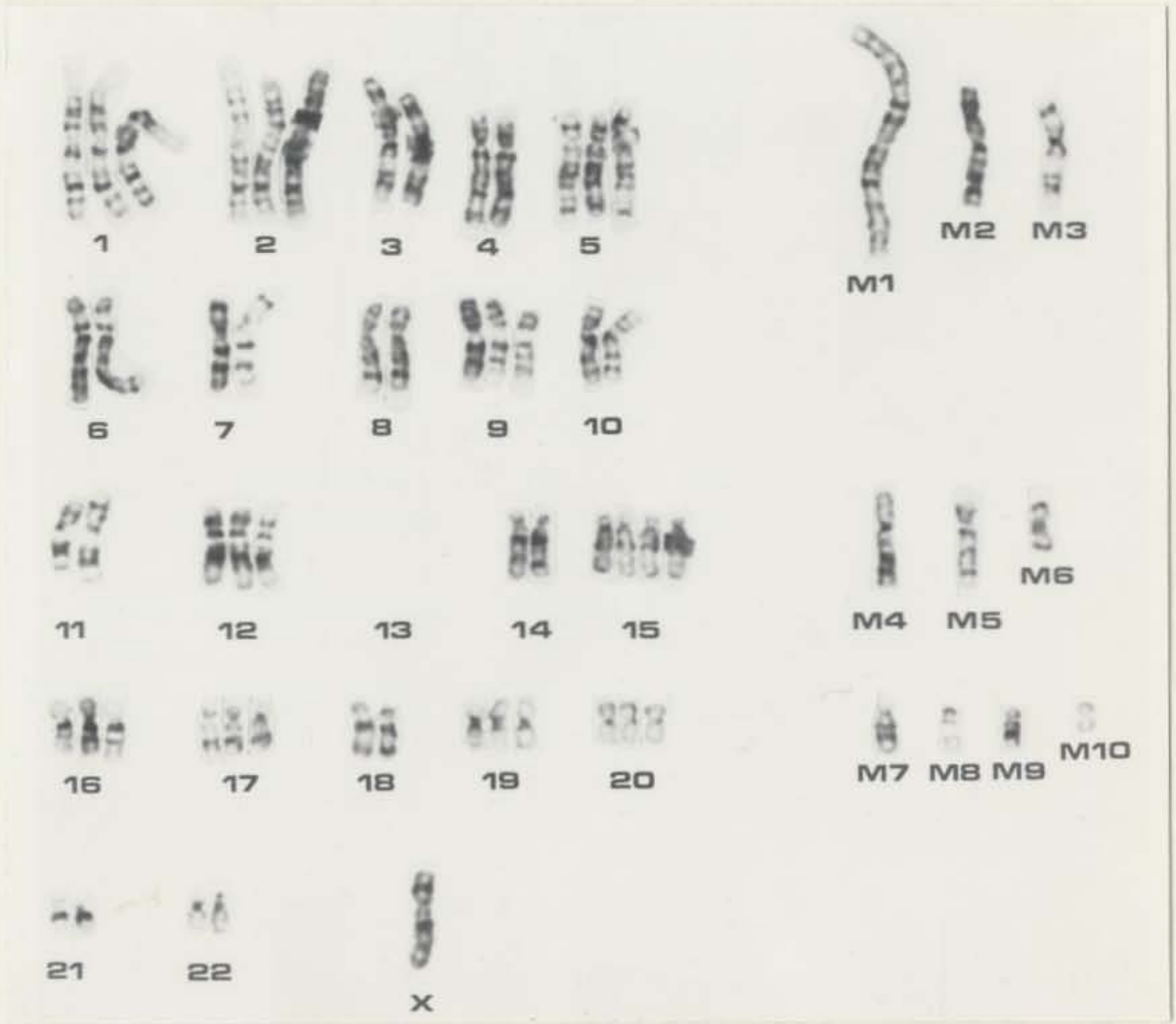

Plate 1. A Giemsa banded karyotype representative of a metaphase cell from the human tumor cell line, HT29 (colonic adenocarcinoma). The chromosome number is 64 and it contains 10 marker chromosomes (M1 to M10).

Marker chromosomes are structurally abnormal chromosomes including dicentric, ring and minute chromosomes.

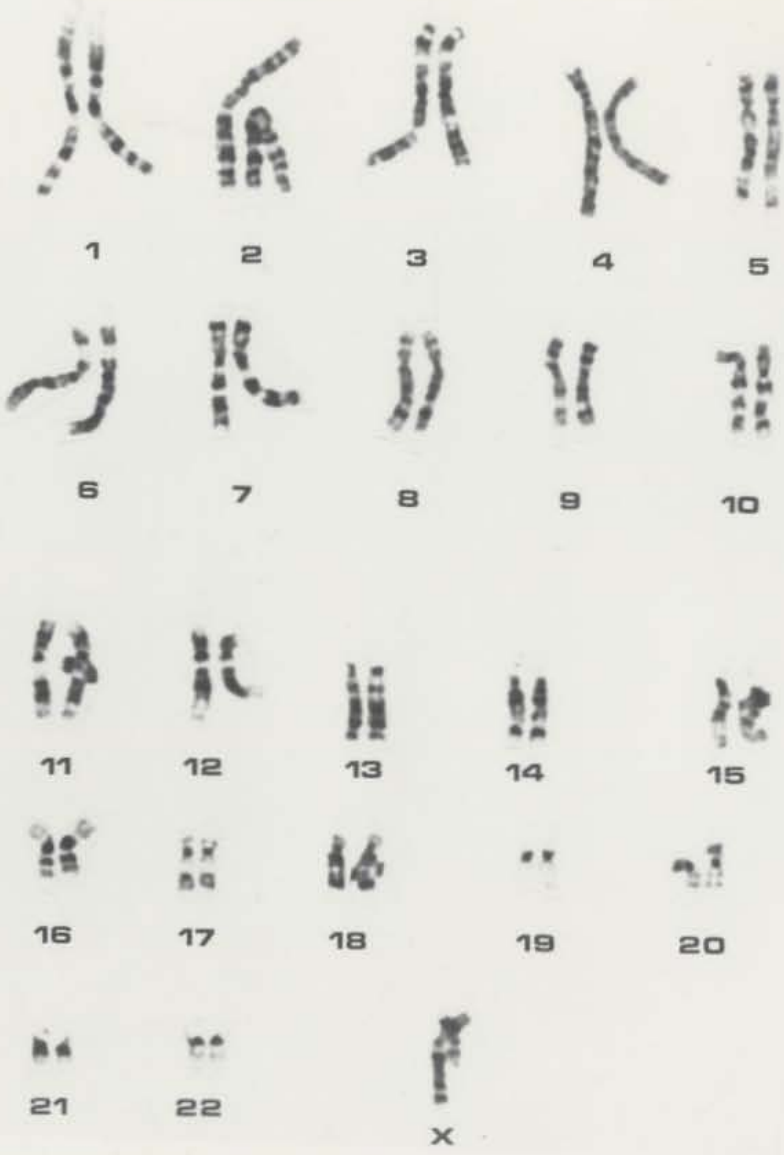

Plate 2. A Giemsa banded karyotype representative of a metaphase cell from the human tumor cell line, LS174T (colonic adenocarcinoma). The chromosome number is 45 and no marker chromosomes were identified.

Plate 3. A Giemsa banded metaphase cell from the human tumor cell line, SKCO1 (colonic adenocarcinoma). Many marker chromosomes have been identified (arrows).

3.2 Screening for CEA by ELISA.

An indirect two stage antibody-enzyme immunoassay was routinely in use for measuring CEA in this laboratory and it was used for screening supernatants from parent cell lines and hybrids (Materials and Methods, Section 2.7.1). A representative standard curve obtained with this assay is illustrated in Figure 2. From this it can be seen that the lowest leval of sensitivity with the assay is 3 ng per ml and that the most accurate range is between 20-100 ng per ml, the linear part of the curve.

In order to maximize the possibility of detection and not miss any potential CEA secreting hybrids, it was decided that the operational definition of CEA production would usually be an absorbance value equal to or greater than the 3 ng per ml CEA standard. On one occasion the initial results for cloning were disappointing in that many of the values were below 3 ng per ml. For this reason a lower cut-off of the BSA control plus two standard deviations was arbitrarily used for selection in order not to miss any potential hybrids in this case (see Appendix II: VIII. ELISA #8).

Note: All ELISA data are in Appendix II.

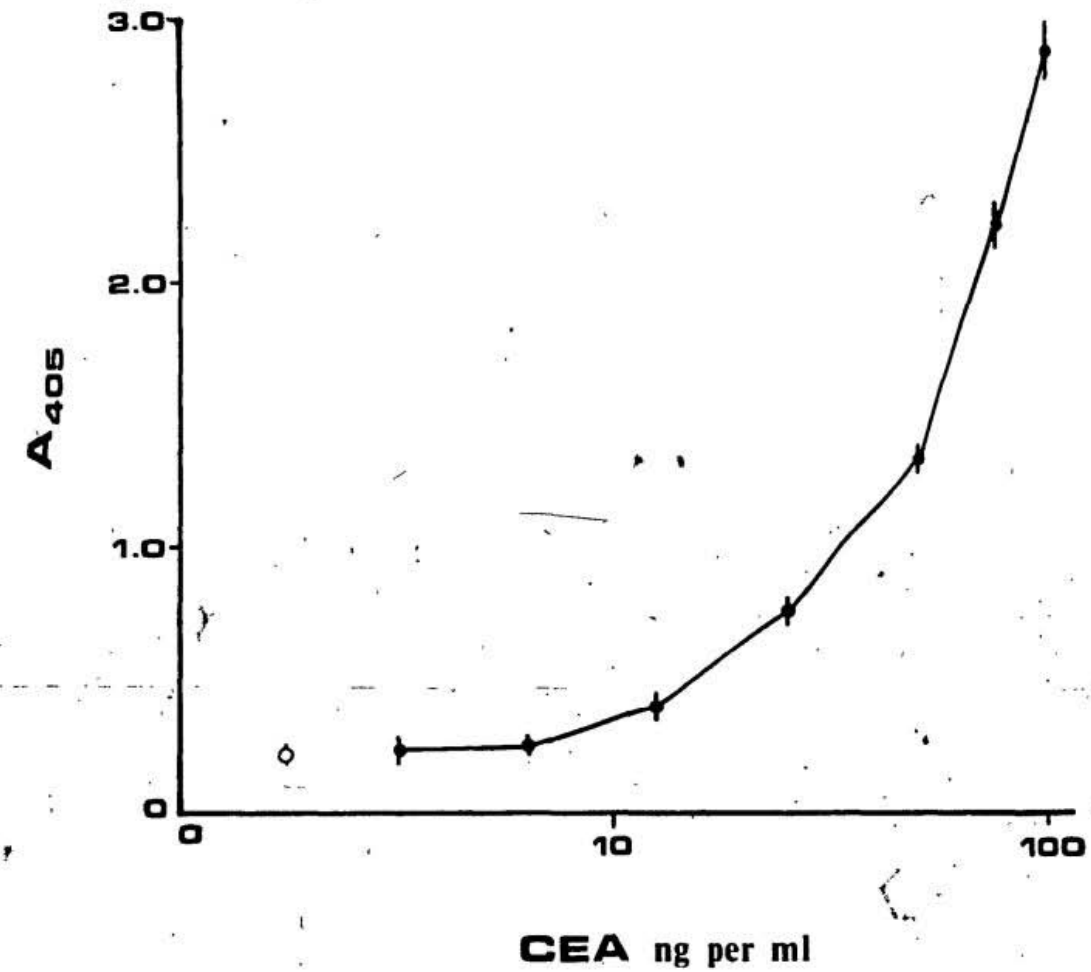

Figure 2. A representative CEA standard curve. Absorbance at 405 nm (ordinate) versus log CEA concentration 3 ng to 100 ng per ml (abscissa). The ● represent the value obtained for each CEA standard and the O represent the value for the PBS/BSA control. RPMI-HAT controls usually gave absorbance values similar to or slightly greater than the PBS/BSA control.

3.3 Production of Human-Mouse Interspecific Hybrids

3.3.1 Testing Ouabain Selection

Before any interspecific fusions were attempted, the HAT-ouabain double selection system was investigated. Previous intraspecific fusions to produce mouse-mouse hybridomas in this laboratory had shown the HAT selection system had ended the growth of NS1 myeloma cells. To demonstrate that the ouabain component of the selection system was capable of killing human tumor cells, LS174T cells were tested against three ouabain concentrations ($1x10^{-4}$ M, $1x10^{-5}$ M, $1x10^{-6}$ M). In addition, the NS-1 cells were also tested in an identical way to ensure that ouabain had no toxic effects on their growth. Cell densities four times the usual plating densities were tested to ensure that ouabain was capable of killing 100% of the human cells used in the fusions. This killing effect was defined as the absence of colonies in 96 well cultures after 30 days incubation. A ouabain concentration of $1x10^{-5}$ M selectively killed 100% of the human tumor cells whereas the mouse cells were unaffected (Table 12). However, for the ouabain concentration of $1x10^{-6}$ M, some viable LS174T cells remained in some of the wells.

Table 12. Effect of different ouabain concentrations on LS174T and NS1.

Ouabain Concentration	Cell Line	Fusion Plating Density (cells per well)	No. Wells containing Viable Colonies*
1x10-4 M	LS174T	1x10^5	0
	NS-1	2x10^4	10
1x10-5 M	LS174T	1x10^5	0
	NS-1	2x10^4	10
1x10-6 M	LS174T	1x10^5	2
	NS-1	2x10^4	10

* A total of ten wells was used for each ouabain concentration.

3.3.2 Fusions with Mouse Suspension Cell Lines (NS1, SP2/0)

The basic technique used for developing interspecific hybrids was suspension fusion with polyethylene glycol (PEG), established in the middle 1970's (Pontecorvo, 1975; Davidson and Gerald, 1976; Hales, 1977; O'Malley and Davidson, 1977). In addition, dimethyl sulfoxide (DMSO) was used with PEG to enhance the fusion process (Norwood, Zeigler, and Martin, 1976). This suspension fusion technique, used successfully in this laboratory to produce mouse-mouse hybridomas, was used initially to try and develop interspecific hybrids between LS174T, SKCO1, HT29, and the mouse myeloma cell line, NS-1. Using the standardized fusion protocol (Materials and Methods, section 2.3.2), ten fusions were performed with no success in obtaining viable hybrids. In view of this, it was decided to work through several fusion parameters systematically including fusion ratios (10:1, 15:1; human to mouse) and fusion plating densities ranging from 3.4x10^4 to 6.6x10^4 cells per well. In addition another mouse cell line, SP2/0, was used as a fusion partner. In the

next nine fusions performed, with the fusing ratio increased two-fold and three-fold respectively, viable colonies were produced in five fusions with LS174T as the human parent cell line.

3.3.3 LS174T Ouabain Resistance

A total of 72 colonies were produced in these five fusions. Characterization included ELISA screening of the spent medium from each colony for CEA activity and Giemsa-11 differential staining of the chromosomes from selected colonies from each fusion to confirm that these colonies were "true hybrids". ELISA screening indicated that all of the colonies were secreting CEA, many having very high levels. Differential staining revealed that out of all of the colonies examined (23/72) none contained mouse chromosomes (Table 13). Both ELISA and G-11 results indicated that the apparent LS174T "hybrids" were in fact either parental LS174T cells or fused LS174T cells which had become resistant to ouabain at 1×10^{-5} M.

Table 13. Giemsa-11 analysis of metaphase chromosomes from selected primary colonies.

Fusion Partners	No. Primary Colonies Screened	Chromosome Origin (Human or Mouse)
LS174TxNS1	5/13	Human
LS174TxSP2/0	3/15	"
LS174TxNS1	3/17	"
LS174TxSP2/0	8/17	"
LS174TxSP2/0	4/10	"

Although earlier experiments (Table 12) had demonstrated that ouabain at 1x10-5 M was toxic to LS174T cells, it was decided to retest ouabain at different concentrations on different plating densities of LS174T cells. Surprisingly, the results indicated that these cells could become drug resistant to ouabain at both concentrations (Table 14). This resistance to ouabain appeared to be independent of cell plating density because the greatest number of ouabain resistant colonies developed at the intermediate concentration, 5x104 cells per well.

Table 14. Summary of LS174T ouabain resistant colonies derived from two ouabain selection experiments

Plating Density * (cells/well)	Number of Resistant Colonies Derived: Ouabain (1x10-4 M)	Ouabain (1x10-5 M)
2.5x104	3	5
5.0x104	6	13
1.25x105	0	12
	9 total	**30 total**

* Cells were aliquotted into 96 well culture plates and a total of twenty wells was used for each parameter tested.

In view of these problems with LS174T, it was decided to omit this cell line from any further fusions.

3.3.4 Evaluation of Different Fusion Parameters

As there had been no production of viable interspecific hybrids, it was decided to make further changes to the fusion protocol. Using the fusion partners (SKCO1, HT29, NS-1, and SP2/O) the following fusion parameters were tested: (1) cell fusion ratio, (2) cell density, and (3) HAT-ouabain selection schedule. Cell fusion ratios of 1:1, 5:1, and 10:1 (human to mouse cell ratio) were individually tested for each fusion partner combination. The plating cell density was increased to 1.25x10^5 cells per well for all twelve fusions to determine if it had any influence on the outcome and PEG 1500 was used as the fusogen. Finally, the HAT-ouabain selection was started 24 hours after the actual fusion process instead of immediately following it. It was believed that this might be important in allowing the fused cells time to "adjust" before the selection pressures were added (Davidson and Gerald, 1976; Gefter, Margulies and Scharff, 1977; Brahe and Serra, 1981). No viable hybrids were obtained in any of the twelve fusions.

To determine whether PEG was a critical factor, the twelve fusions were repeated using PEG 4000 instead of PEG 1500. A viable hybrid was developed between HT29 and NS-1. This was confirmed to be a true interspecific hybrid by the differential G-11 staining technique. However, preliminary screening of the spent medium for secreted CEA by ELISA failed to demonstrate any CEA production.

At this point it was considered that the failure to obtain hybrids might be due to some incompatibility of the parent cell lines; it seemed less likely to be due to the fusion method itself. It was decided to try

fusing the human cell lines, HT29 and SKCO1, with new mouse cell lines using the same basic method. The new mouse cell lines RAG (mouse renal adenocarcinoma), PG19 (mouse melanoma) and STO (mouse embryonic fibroblast) were chosen for several reasons. First, all three were HAT sensitive. Second, all three were cells which grew as monolayers. Finally, with the exception of the STO cell line, they had been successfully used in previous interspecific human-mouse fusions by other investigators. The fusion parameters were standardized as follows: (a) PEG 4000, (b) cell ratio 1:1, (c) cell density 2.5 x 104 cells per well, and (d) HAT-ouabain selection 24 hours after fusion of cells with PEG. With the exception of PG19, the number of viable hybrids increased dramatically with the new mouse fusion partners (Table 15). The cell morphology of the resultant hybrids is compared to the parent cells in Plate 4 (HT29, RAG, STO, HT29xRAG and HT29xSTO) and Plate 5 (SKCO1, RAG, STO, SKCO1xRAG and SKCO1xSTO).

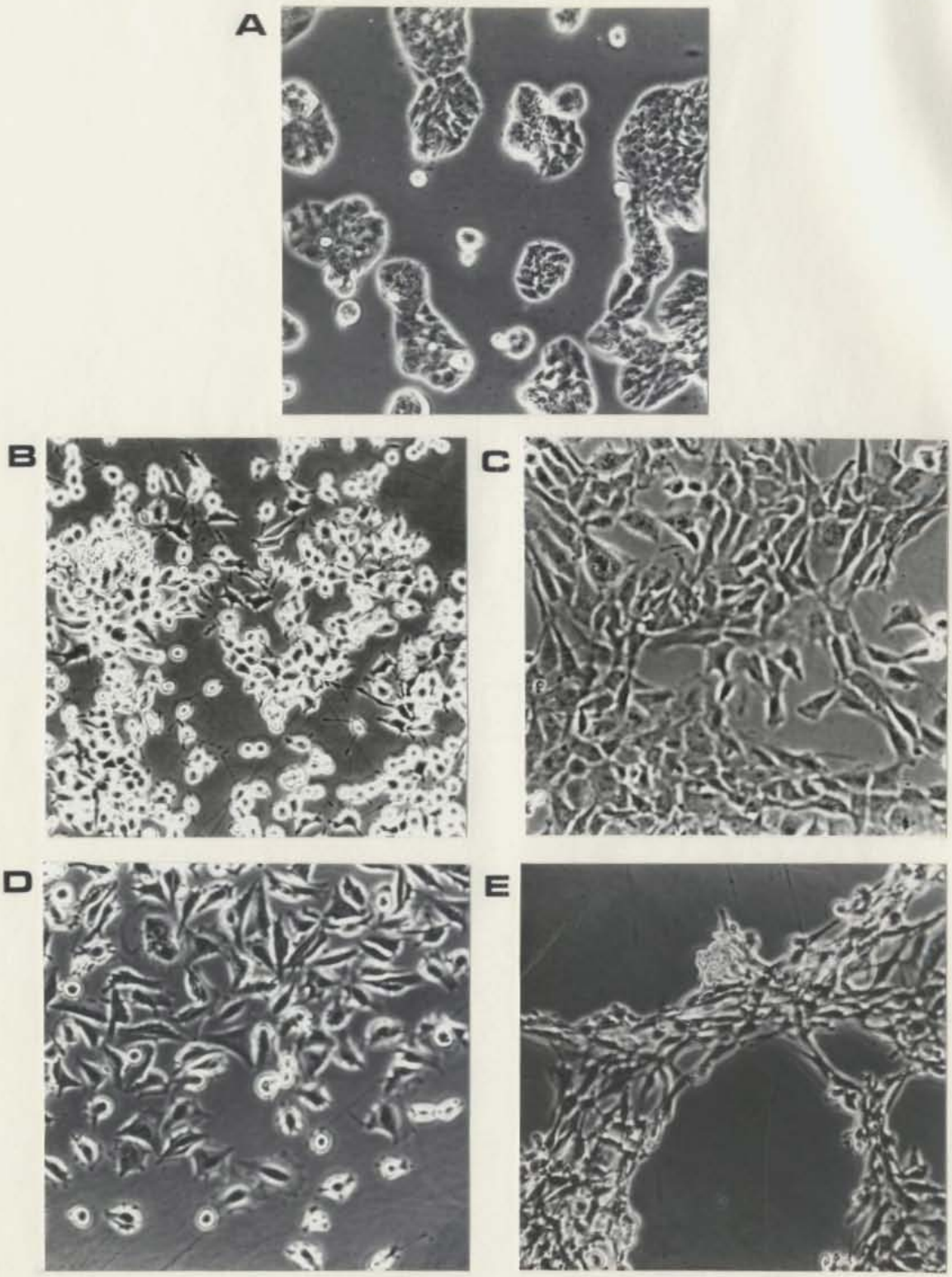

Plate 4. A comparison of cell morphology between HT29 (A), RAG (B), STO (C), HT29xRAG (D) and HT29xSTO (E) (Magnification 40x).

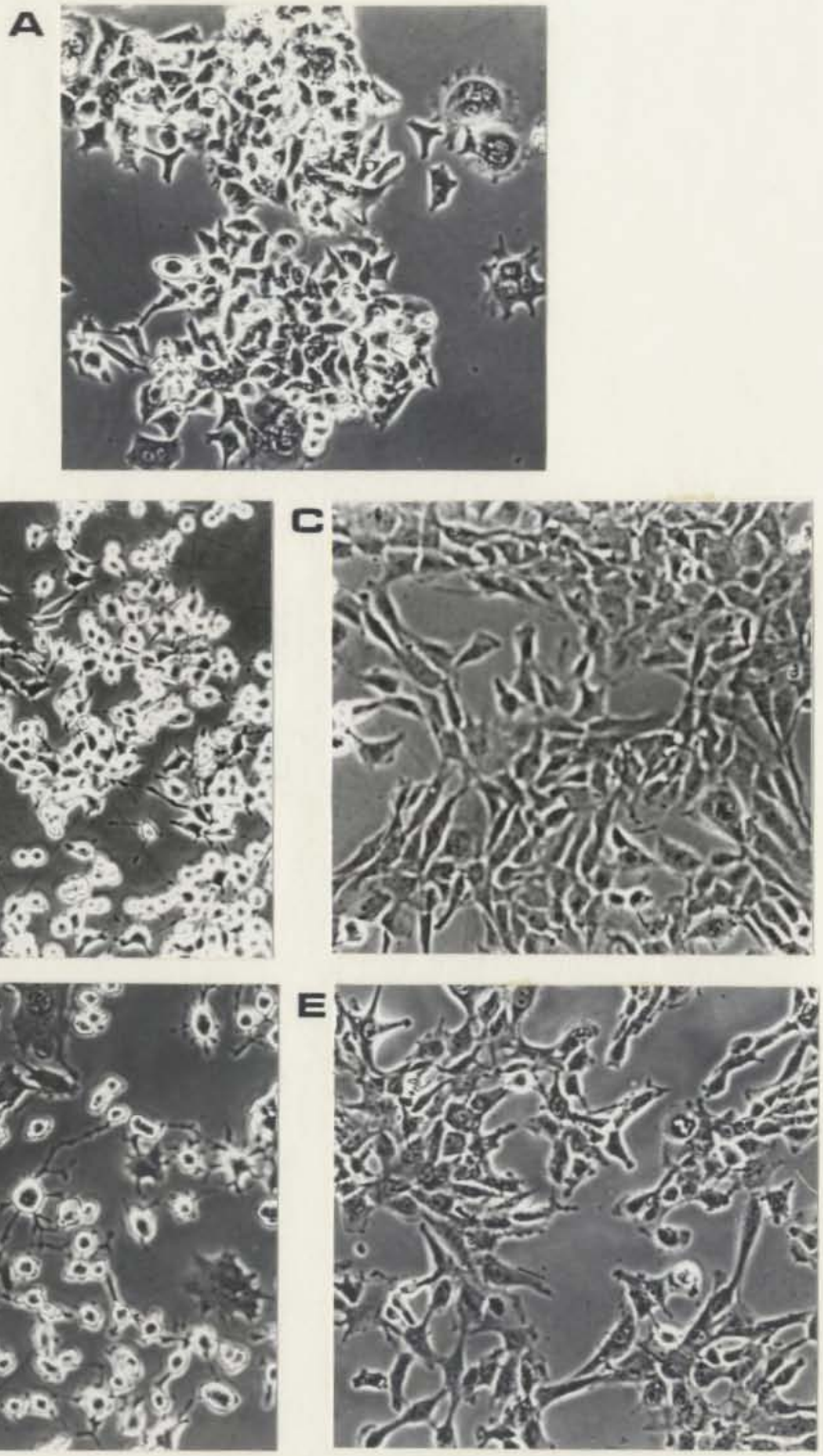

Plate 5. A comparison of cell morphology between SKCO1 (A), RAG (B), STO (C), SKCO1xRAG (D) and SKCO1xSTO (E) (Magnification 40x).

Table 15. Summary of interspecific fusions.

Fusion Type	Fusion Partners Human	Mouse	No. Fusions Performed	No. Hybrid Colonies
1. Monolayer-suspension	HT29	NS1	8	1
		SP2/0	7	0
	SKCO1	NS1	11	0
		SP2/0	7	0
	LS174T	NS1	6*	0
		SP2/0	4*	0
2. Monolayer-monolayer	HT29	RAG	1	185
		STO	1	130
		PG19	1	0
	SKCO1	RAG	1	25
		STO	1	3
		Total	48	344

* LS174T ouabain resistant colonies were derived in 2/6 LS174TxNS1 fusions and 3/4 LS174TxSP2/0 fusions.

3.4 Characterization of Interspecific Hybrids

3.4.1 Primary Analysis of Hybrids for CEA Production by ELISA

Preliminary screening of the original colonies produced in the last four fusions resulted in the selection of 49 out of 244 colonies (Table 16).

Table 16. Summary of ELISA screening of original hybrids.

Parent Cell Lines	No. Colonies * Screened/ Total Colonies Obtained	No. of CEA Positive Hybrids	No. of Primary Colonies Selected For Cloning
HT29 NS1	1/1	0	0
HT29 RAG	179/185	30	18
SKCO1 STO	3/3	0	2 +
HT29 STO	41/89 a	19	9
SKCO1 RAG	20/23 b	3	1
Total	**244/301**	**49**	**30**

* The most vigorously growing colonies were selected for screening.
\+ Because SKCO1 is a high CEA expressor/secretor it was decided to clone 2 hybrids (F3.2 and F3.3) to ensure potential hybrids were not missed.
a 41/130 original HT29xSTO colonies died.
b 2/25 original SKCO1xRAG colonies died.

All of the 49 hybrids selected as being CEA positive had low absorbance values. In order to try and enrich for CEA production, 28 with the highest values (plus F3.2 and F3.3) were selected for cloning

(Appendix I, Table 1). Subsequently, five hybrid colonies from the HT29xSTO fusion died in culture. Twenty-three of the 25 remaining primary hybrids were cloned successfully by limiting dilution (Materials and Methods, Section 2.4) resulting in the isolation of 131 clones (Appendix I, Table 2). Subsequently, these clones were transferred to 24 well culture plates, grown to confluence and spent medium samples from each tested for CEA by ELISA. Twenty-four out of 131 primary clones were found to be greater than the 3 ng CEA standard (Table 17).

Table 17. Summary of ELISA screening of primary clones.

Parent Cell Lines	No. of Primary Clones Obtained	No. of CEA Positive Primary Clones	No. of Primary Clones Selected for recloning
HT29 RAG	117	20	16
SKCO1 STO	2	1	0
HT29 STO	8	3	0
SKCO1 RAG	4	0	0
Total	**131**	**24**	**16**

Primary cloning did not result in hybrid colonies which were higher CEA secretors and because of this a further attempt to enrich for CEA producing hybrids was made by re-cloning the highest 16 based on the absorbance values obtained in ELISA (Appendix I, Table 3).

Subcloning the HT29xRAG primary clones resulted in the isolation of 126 secondary clones (Appendix I, Table 4), 104 of which were found to be CEA positive by ELISA. Although the number of potential CEA producing hybrids had increased, CEA secretion was still at the threshold of sensitivity of the ELISA. Prior to this, most of the cultures tested for secreted CEA were supernatant samples removed from confluent 24 well cultures. In order to increase the chances of confirming CEA production ten HT29xRAG secondary clones were selected for further evaluation (Appendix I, Table 5). Each subclone was dispensed into 2x75 cm^2 flasks at a concentration of $1x10^5$ viable cells in 10 ml of RPMI-HAT medium and on days 6 and 9 medium from each flask was assessed for CEA by ELISA. In addition, the parent cell line, HT29, was also set up and assessed in the same manner. None gave values greater than the 3 ng per ml CEA standard.

3.4.2 Secondary Analysis of Selected Hybrids for CEA.

To investigate any potential CEA expression/secretion by hybrids in more detail, a number of approaches were taken. Since CEA expression and release of CEA into the culture medium might not correlate, immunochemical evaluation with monoclonal and polyclonal anti-CEA antibodies (Materials and Methods, Section 2.8.2) and ELISA for CEA in membrane/cytosol fractions of selected hybrids was undertaken (Materials and Methods, Section 2.5).

3.4.2.1 Immunoperoxidase Assay

Selected hybrids were tested for cell surface or cytoplasmic expression of CEA by the two stage indirect immunoperoxidase technique (Materials and Methods, Section 2.8.2). The anti-CEA antibodies used were monoclonal antibody 11-285-14, and a polyclonal antibody, DAKO. Smears were prepared from each fusion and a summary of the immunocytochemical data is provided (Table 18).

Table 18. Summary of immunocytochemical results.

Cells Tested	Reactivity with 11-285-14 (% cells stained)*	Reactivity with DAKO anti-CEA (% cells stained)*
1. Human cell lines **		
HT29	4	NT
SKCO1	80	NT
LS174T	60	NT
2. Mouse cell lines		
RAG	0	0
STO	0	0
3. Hybrid cells		
HT29xRAG (29 tested)	0	0
HT29xSTO (4 tested)	0	0
SKCO1xRAG (15 tested)	0	0
SKCO1xSTO (2 tested)	0	0
Total tested = 50		

* The percentages were assessed qualitatively by visualizing the number of positively stained cells.
** Data based on repetitive testing (Ford et al., 1987).
NT-not tested

3.4.2.2 Membrane and Cytosol Fractions

Four hybrids were selected for determination of CEA levels in the membrane and cytosol fractions. Cells were grown to confluence in eight 75 cm^2 tissue culture flasks, trypsinized, lysed by ultrasonication and separated into membrane and cytosol fractions by centrifugation (Materials and Methods, Section 2.5). Subsequently, these were assessed for CEA content by ELISA and the results are summarized (Table 19).

Table 19. Summary of ELISA for CEA in subcellular fractions.

Cells Tested	Total Cells ($x10^6$)	CEA (ng per 10^6 cells) Membrane	Cytosol
1. Human cell lines *			
SKCO1	108.7	119	248
LS174T	172.4	14	12
HT29	62.8	7	5
2. Mouse cell lines			
RAG	157.0	< 0.1	< 0.1
STO	102.0	< 0.1	< 0.1
3. Hybrid cells +			
SKCO1xRAG			
F5.2	20.6	0.42	< 0.1
F5.19	10.5	0.27	0.27
HT29xRAG			
F2.131.D2C4	31.2	0.20	< 0.1
F2.179.A9C6	12.3	0.71	0.25

* These lines had been previously characterized in this laboratory and this data supplied by C.H.J. Ford.

\+ The hybrid cell numbers used in this experiment were lower than the parent cell lines because the cell yield from 8x75 cm^2 flasks was much lower.

3.4.2.3 Concentrated Spent Medium

In order to determine whether the low absorbance values, and hence borderline CEA levels, in culture supernatants might reflect very low production, spent medium from 6 hybrids (HT29xRAG, 2 hybrids; SKCO1xRAG, 2 hybrids; SKCO1xSTO, 2 hybrids), HT29 and RPMI-HAT medium were concentrated 5x and assayed for CEA by ELISA. Absorbance values were, again, low indicating borderline CEA levels at the lower end of the CEA standard curve.

3.4.3 Chromosomal Analysis of Selected Hybrids

During secondary analysis of selected hybrids for CEA production, it was decided to characterize selected hybrids for human chromosome content to gain experience with the techniques (Materials and Methods, Section 2.7) in case any definite CEA expressing/secreting hybrids were obtained.

3.4.3.1 Giemsa Alkaline Differential Staining.

Initial characterization of the ten HT29xRAG subclones for human chromosomes was undertaken by performing both staining techniques on the same slide (i.e. Giemsa-11 differential staining, destaining, and Giemsa-trypsin banding on the same slide). However, due to poor quality of the chromosomes after Giemsa banding, it was decided to stain slides separately. That is, half of the slides prepared from a chromosome harvest were stained by the differential staining method and the other half were banded by the Giemsa-trypsin banding method. The G-11 stain enables differentiation between human and mouse chromosomes by

color (Plate 6)

The approximate number of human chromosomes in each subclone, determined by analysing differentially stained metaphases, ranged from 7-18 for F2.131.D2C4 to 26-35 for F2.179.B7D8 (Table 20).

Table 20. Giemsa-11 analysis of ten HT29xRAG subclones.

Subclone Code Name	No. of Metaphases Screened	Human Chromosome Range
F2.169.E9E1	59	14-19
F2.169.E9E4	40	10-14
F2.169.E9F1	88	10-19
F2.169.E9F7	59	16-23
F2.179.B7D8	89	26-35
F2.131.D2F2	44	21-32
F2.179.B7E8	85	23-31
F2.131.D2C4	48	7-18
F2.179.A9C6	105	18-22,25-35
F2.130.F9D2	38	21-26

3.4.3.2 Giemsa Banding

Giemsa banding was undertaken in order to determine the specific human chromosome content of selected hybrids (HT29xRAG, F2.179.A9C6; SKCO1xRAG, F5.3). In addition the mouse cell line, RAG, was G-banded (Plate 7) because it contained bi-armed marker chromosomes which were similar to some of the human chromosomes. Problems encountered included difficulty in identifying all the human chromosomes in hybrid cells and the presence of many chromosomes in individual hybrid cells. Despite these problems representative cells for both hybrid cell types were G-banded and human chromosomes were identified as illustrated in Plates 8 and 9.

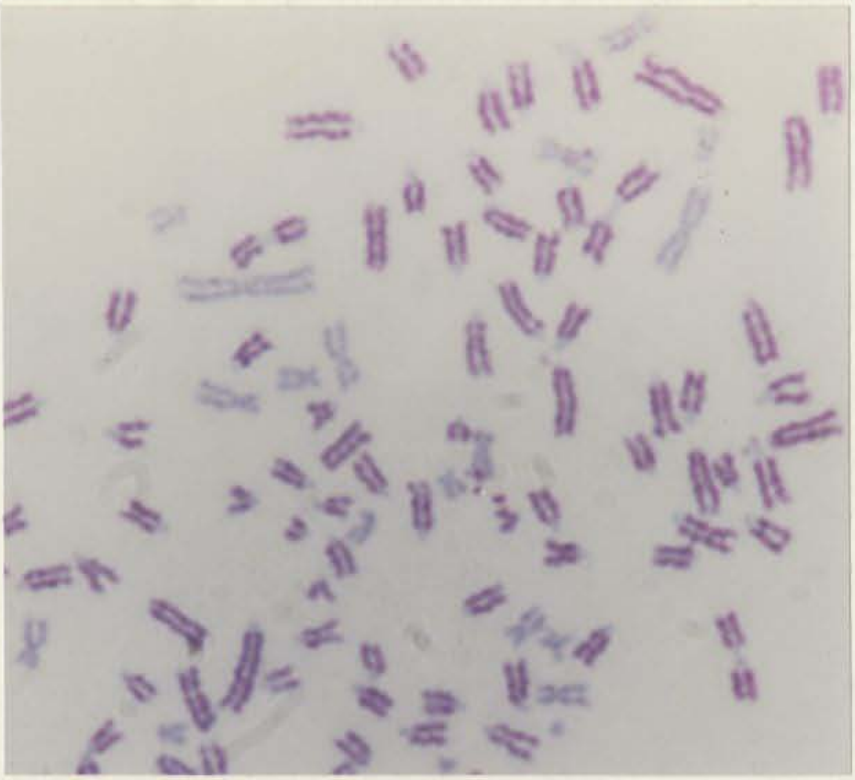

Plate 6. Differential staining of human and mouse chromosomes by the G-11 staining technique (Alhadeff et al., 1977). This is a partial metaphase from an HT29xRAG hybrid. Mouse chromosomes stain magenta red whereas human chromosomes stain pale blue.

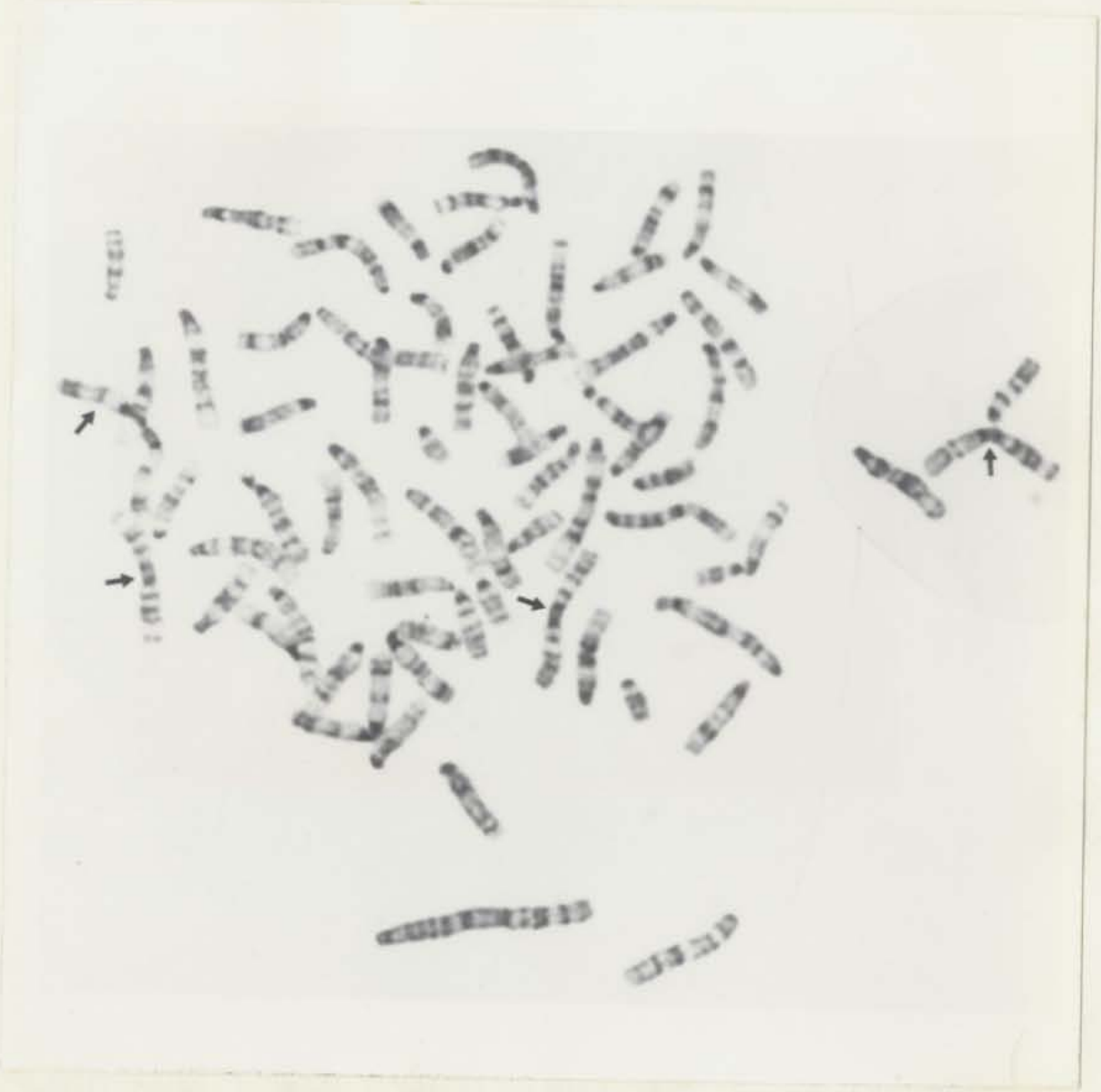

Plate 7. A Giemsa banded metaphase cell from the mouse tumor cell line, RAG (renal adenocarcinoma). The biarmed chromosome markers are identified by arrows.

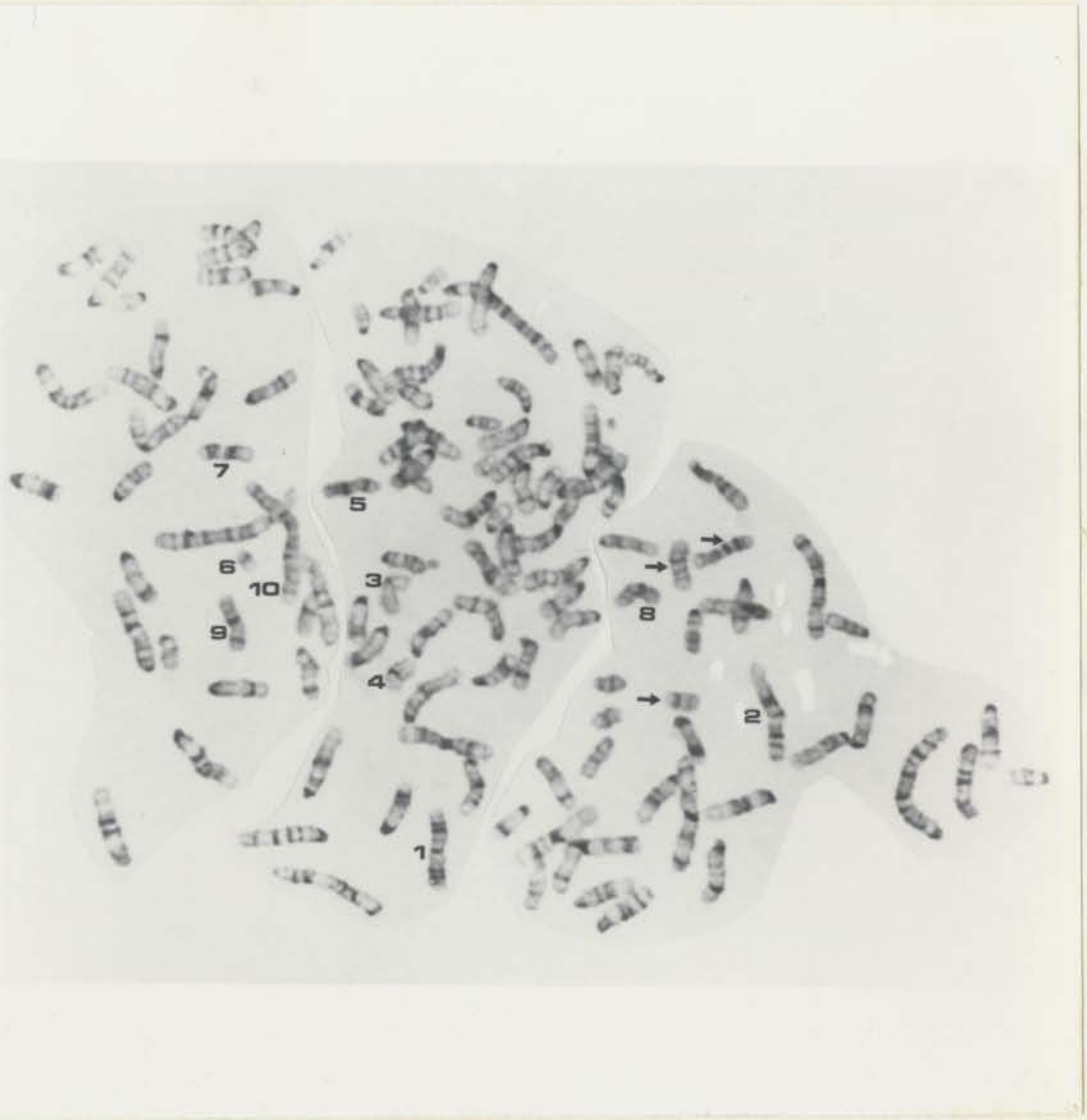

Plate 8. A Giemsa banded hybrid metaphase cell from HT29xRAG (F2.179.A9C6) showing human chromosomes (normal and marker) numbered from 1 to 10. In addition, some other chromosomes are indicated by arrows which are believed to be of human origin. In Giemsa banded hybrid metaphases the centromeres of mouse chromosomes stain more intensely than human centromeres.

Plate 9. A Giemsa banded hybrid metaphase cell from SKCO1xRAG (F5.3) showing human chromosomes (normal and marker) numbered from 1 to 11. In addition, some other chromosomes are indicated by arrows which are believed to be of human origin.

Chapter 4
DISCUSSION

4.1 Production of Interspecific Hybrids

Chromosomal assignments for a number of human cell surface molecules with restricted tissue distribution have resulted from serological analysis of human-rodent somatic cell hybrids. To date cell surface antigens have been mapped to human chromosomes 1, 3, 6, 7, 11, 12, 15, 17, X and Y by immunological analysis of hybrids with monoclonal antibodies identifying cell surface antigens (de la Chapelle, 1986). Studies such as these have provided the background for the investigations undertaken in this thesis.

There are several important factors that should be considered for any gene mapping study which uses somatic cell genetic techniques. First, the human parent cell should express the tissue-restricted marker. Second, since characterizing hybrids will involve chromosome analysis, it is preferable that a human cell line with as near a normal karyotype as possible is used as the parent cell line because the presence of markedly rearranged chromosomes can make a definitive gene assignment difficult. Another important factor for the study of any tissue-restricted antigen is the choice of rodent parent, since the rodent cell must allow expression of the human gene. Repression of human gene expression can be a common occurrence in interspecific hybrids (Ringertz and Savage, 1976; Tunnacliffe and Goodfellow, 1984).

4.1.1 Selection of Human Parental Cell Lines

The human cell lines, LS174T, SKCO1 and HT29 were chosen as fusion partners for studying the expression of the tumor-associated antigen, CEA, because each cell line had been previously shown by other investigators to secrete the tumor associated marker in sufficient quantities to be detected in immunological assays. Typical values for CEA secretion, for the HT29 cell line, ranged from 17 ng per ml after 7 days in culture to 300 ng per ml after 29 days (Egan and Todd, 1972). Shi and colleagues found that all three lines expressed and secreted measurable quantities of CEA (Shi et al., 1983; Table 8). Their results were obtained using a commercially available radioimmunoassay for CEA based on a polyclonal anti-CEA antibody to which a correction factor had to be applied for very low values.

Results obtained in this laboratory with the ELISA described in the Materials and Methods (Section 2.8.1) also clearly demonstrated membrane and cytoplasmic CEA (Table 19), although the exact values obtained were different from the results reported by Shi et al., (1983), possibly due to differences in assay techniques. In addition, immunocytochemical and radiolabelled antibody binding studies performed with the monoclonal anti-CEA antibody, 11-285-14, had confirmed the CEA expression of these cell lines (Ford et al., 1987; Table 8). Given these data, the selection of these lines as fusion partners was believed to be a reasonable choice.

4.1.2 Fusion Efficiency

Several measures have been used to define successful fusions in somatic cell hybridization experiments. One measure is "fusion efficiency". This is defined as the percent of nuclei in bi and polynucleate cells minus the percent in control cultures not exposed to PEG (Okada and Tadokaro, 1962) and it is calculated by counting the number of hybrid cells present after treatment with PEG. Heterokaryons are distinguished from homokaryons by staining the cells with hematoxylin and eosin. Another measure, referred to as the "fusion index", is defined as the percentage of single cells of the original untreated population that have entered into a multinucleate fusion product. It is calculated by summation of the nuclei present in cell fusion products after treatment, followed by division of the total number of nuclei present in the observed sample (Hansen and Stadler, 1977). Another measure used is the "effective mating rate" which indicates the frequency of formation of viable fusion products (Davidson and Ephrussi, 1970). It is obtained by counting the observed number of viable hybrid colonies and was used to assess the success of fusions in the results reported in this thesis.

A. Monolayer-Suspension Fusions

Using the "suspension" fusion technique (Materials and Methods, Section 2.3.2) attempts were made to produce viable, stable hybrids between the human colorectal carcinoma cell lines (LS174T, SKCO1 and HT29) and the mouse myeloma cell lines (NS1, SP2/0). The mouse myeloma cell lines were selected as fusion partners because in the past

development of mouse-mouse hybrids with them had been successful and it was thought that the myeloma cells might permit expression of CEA. This was felt to be important because another investigator, trying to map the CEA gene(s) by somatic cell genetic methods, had developed interspecific human-mouse hybrids between the human colorectal cancer line, LS174T and two mouse cancer cell lines, RAG and PG19. By using two goat anti-CEA polyclonal antisera, in a competitive radioimmunoassay (RIA) to screen for positive CEA-producing interspecific hybrids, Sheer was able to identify some low CEA producing hybrids. However, her attempts to isolate higher CEA-producing clones by fluorescence-activated cell sorting were unsuccessful. It was suggested, although not proven, that the apparent reduction in CEA production in the interspecific hybrids was due to repression of the CEA gene(s) by the mouse genome (Sheer, Brown and Bobrow, 1982).

In the present study a total of 46 fusions were performed between these cell types (Table 15) and only one viable hybrid was derived. Several parameters were evaluated including cell fusion ratio (1:1, 5:1, 10:1 and 15:1 human/mouse), cell density. (2.5×10^4 to 1.25×10^5 cells per well) and molecular weight of PEG (1500 vs 4000) to see if the fusion efficiency would increase. Because the changes to the standard fusion method resulted in no significant improvement in the number of viable hybrids produced, it was decided that this result might not be a function of the fusion method but rather of the "incompatibility" of the parent cell lines (i.e. monolayer x suspension).

B. Monolayer-Monolayer Fusions

After investigating several different parameters in the monolayer-suspension fusions with limited success, it was decided to try different mouse cell lines as fusion partners. The three lines (RAG, STO and PG19) were selected primarily for the reasons outlined (Results, Section 3.3.4). Fusions between cells with similar growth properties have been shown to result in a higher fusion efficiency (Davidson and Gerald, 1976; O'Malley and Davidson, 1977; Anders, Wierda, Nienhaus and Idenburg, 1978; Brahe and Serra, 1981). Using the "suspension" fusion technique (PEG 4000, cell fusion ratio 1:1, fusion density 2.5×10^4 cells per well) 5 fusions were performed with the number of colonies obtained ranging from 0 to 285.

4.1.3 LS174T Ouabain Resistance

The production of LS174T ouabain resistant colonies was unexpected because earlier results (Table 12) had clearly shown that the ouabain concentration of 1×10^{-5}M was capable of killing 100% of the LS174T cells. Ouabain sensitivity of LS174T cells has also been demonstrated previously by others. For example, it was reported that sparsely populated and confluent cultures of LS174T exposed to 1×10^{-5}M ouabain died within 5 and 18 days respectively (Sheer, 1980). Re-evaluation of the ouabain selection on LS174T was undertaken to see if the resistance was dependent on the cell density per microtiter well. The cells were resistant to ouabain at both the selection concentration (1×10^{-5} M) and 10x the selection concentration. Interestingly, Sheer did report that three

hybrid clones between LS174T and PG19 (mouse melanoma) had no human chromosomes (Sheer, 1980). This suggests that these clones were not "true hybrids" but might have been LS174T ouabain resistant colonies.

4.1.4 Other Fusion Studies

Studies on the somatic cell genetics of mammals have been limited in many cases by the ability to develop viable hybrid cells. In some cases it has been possible to increase the efficiency of hybridization by altering the conditions of culture or the ratios of the parental cells (Davidson and Ephrussi, 1970).

Factors such as the type of fusion technique and PEG have a significant effect on fusion efficiency. There is a marked effect of PEG concentration on cell hybridization, and there seem to be inherent differences between cells in terms of the extent of cell fusion induced by PEG. PEG will efficiently fuse mammalian cells over a narrow range of concentrations (45-55% PEG) above which or with prolonged exposure the fusion index drops rapidly (Davidson and Gerald, 1976). Several investigators have demonstrated that PEG is more effective in promoting high rates of cell fusion between substrate attached mammalian cells than between cells in suspension. It has been suggested that this is the result of PEG toxicity. However, it has also been shown that the efficiency of mouse myeloma-mouse myeloma fusions decreased dramatically when PEG concentrations greater than 40% were used (Gefter, Margulies and Scharff, 1977). This may be an explanation for the low fusion efficiency between human colorectal-mouse myeloma cells found in the present study.

This could be tested in future by determining the fusion efficiency between these cell types with lower PEG concentrations.

Since many cell types cannot attach to a substrate, e.g., myeloma cells, and the use of suspension cell types as fusion partners in hybridization experiments is often required, problems arise with respect to getting these cells in contact with cells grown in monolayer. Several groups have demonstrated that when they used a suspension fusion technique referred to as "pancake fusion", the fusion index for monolayer cell type x suspension cell type fusions could be improved (O'Malley and Davidson, 1977; Anders et al., 1978). This method could be investigated to see if the fusion efficiency for the monolayer-suspension fusions between human colorectal lines (HT29, LS174T and SKCO1) and mouse myeloma lines (NS1 and SP2/0) can be improved. Another group that obtained hybrids from fusions between monolayer cells and suspension cells with a different monolayer fusion technique, suggested that the frequency of hybrid colonies varied greatly, depending on the cell line used (Brahe and Serra, 1981) and this is another possible explanation for the low fusion efficiency found in the present study.

Another possible factor which may affect the fusion efficiency, but was not evaluated in the present study, is the use of feeder layers. However, since previous mouse-mouse fusions, with NS1 or SP2/0 as fusion partners, have been performed successfully without feeder layers and other investigators have not reported the use of feeder layers for the growth of interspecific hybrids, they may not provide any clear advantages.

4.2 Characterization of Interspecific Hybrids

The following human and mouse cell combinations: HT29xNS1, HT29xRAG, HT29xSTO, SKCO1xRAG and SKCO1xSTO were isolated by HAT-ouabain double selection and then characterized immunologically for CEA expression/secretion. In addition, on the presumption that CEA producing hybrids would be identified, several hybrids were characterized for human chromosome content to ensure that the chromosome analysis techniques, Giemsa differential staining and Giemsa banding, could be used successfully to identify human chromosomes in these cells.

4.2.1 Immunological Characterization

The antibodies used for recognizing CEA were the mouse monoclonal antibody, 11-285-14, and the rabbit polyclonal antibody, DAKO. Characterization of 11-285-14 has shown that it reacts with affinity purified CEA, colonic, gastric and mammary carcinomas, fetal colonic tissue and gastrointestinal or tonsilar epithelium (Gatter, Abdulaziz, Beverly, Corvalan, Ford, Lane, Mota, NAsh, Pulford, Stein, Taylor-Papadimitriou, Woodhouse and Mason, 1982). It also lacks reactivity with nonspecific cross-reacting antigen (NCA) and most normal tissues (Woodhouse, 1982). The commercially available rabbit anti-CEA (DAKO) antibody, without further absorption, is known to cross-react with NCA and biliary glycoprotein (BGP).

A. Primary Screening

There was no previous data on levels of antigen that might be secreted by definitely positive CEA hybrid cells and it was for this reason that a 3 ng cut-off was used to select for any potential CEA positive hybrids. Primary screening and cloning of hybrids failed to enrich for a subpopulation of cells secreting higher levels of CEA. In retrospect, the threshold of 3 ng used for selecting potential secreting hybrids may have been too low and in future studies it may be more productive to select a higher cut-off to select those hybrids definitely secreting CEA.

B. Secondary Screening

Because the CEA positive interspecific hybrids identified by ELISA were borderline positive it was decided to evaluate CEA expression/secretion in selected hybrids by immunocytochemical analysis for surface expression of CEA (immunoperoxidase assay); measurement of CEA in subcellular fractions (cytosol and membrane) and measurement of CEA in concentrated spent medium.

Methanol-fixed cell smears of selected hybrids were screened separately using both the monoclonal antibody, 11-285-14, and the polyclonal anti-CEA antibody, DAKO, for surface and cytoplasmic expression of CEA. Based on the results (Table 18) none of the hybrids were positive for CEA. Measurement of CEA in subcellular fractions from both the human and mouse parent cell lines and selected hybrids indicated that

the four SKCO1xRAG and HT29xRAG hybrids contained minimal levels of CEA compared to the human parent cells (Table 19). However, one HT29xRAG hybrid (F2.179.A9C6) did have a higher membrane value than any of the others and it might be of interest to extract other hybrids for further assessment. The mouse parent cells, RAG and STO, were negative for CEA. Previous work had also indicated that cell sonicates of LS174TxRAG and LS174TxPG19 hybrids contained no significant levels of CEA (Sheer, 1980).

In addition, spent medium from several hybrids (HT29xRAG, SKCO1xRAG and SKCO1xSTO) were concentrated ten fold and analyzed by ELISA for CEA. Results indicated no increase in CEA values. It appears that if CEA is being produced, then minimal amounts are being secreted. Another possibility, originally suggested by Sheer, is that these hybrids could be producing an abnormal CEA glycoprotein and the anti-CEA antibody used for detecting CEA might not detect the abnormal CEA (Sheer, 1980). The antigenic determinant recognized by our monoclonal antibody, 11-285-14, might be altered or destroyed in the abnormal CEA. Therefore, future investigations could include testing hybrids with a panel of monoclonal anti-CEA antibodies, each recognizing different CEA epitopes (protein and carbohydrate), to confirm that the levels of CEA detected with the 11-285-14 antibody are valid reflections of the hybrid cells.

4.2.2 Chromosomal Analysis

A. Parent Cell Lines

There have been only a few karyotypic studies performed on the three colorectal cancer cell lines, LS174T, HT29, and SKCO1. For those that have been karyotyped, most consist of near triploid or higher chromosome numbers with many structurally abnormal chromosomes. The American Type Culture Collection (ATCC) has reported the following chromosomal results (Hay, Macy, Hamburger, Weinblatt, and Chen, 1983):

(1) LS174T - Modal number, 45 XO. Chromosome range, 36-46. No apparent marker chromosomes.

(2) SKCO1 - No modal number given. The chromosome number ranged from hypertriploid to hypotetraploid with chromosome abnormalities including dicentrics, minutes, rings, secondary constrictions, and large submetacentric markers.

(3) HT29 - No modal number given. The chromosome number ranged from hypo- to hypertriploidies with chromosome abnormalities including dicentrics, acrocentric fragments, minutes, secondary constrictions, large submetacentric and meta- or polycentric markers.

Other independent studies show similar karyotypic results for LS174T. Several groups reported that a majority of the cells displayed 44 or 45 chromosomes with a modal number of 45, had no abnormal chromosomes and the missing chromosomes tended to be the sex chromosomes (Tom et el., 1976; Rutzky et al., 1980). Sheer reported that the chromosomal modal number was 48 with a range of 46-53 in 10 cells analyzed, every cell was trisomic for chromosome 7, and some cells had additional copies

of chromosome 1, 13, and 15 (Sheer et.al.,1982). At present there are no published karyotypes for HT29 and SKCO1, however, Chen and his colleagues are currently karyotyping both of these cell lines (personal communication).

Karyotype studies were performed on the three cell lines using conventional chromosome harvesting and Giemsa banding techniques to obtain metaphase cells and to band chromosomes. The mitotic index tended to be low for these lines and the quality of the Giemsa banded chromosome spreads was often poor. This was usually indicated by highly condensed chromosomes, a high percentage of overlapping chromosomes and chromosome fuzziness. The chromosome ranges were determined for each cell line by counting approximately 100 metaphase cells. The cell line, LS174T, was near diploid in chromosome number (43-47) whereas the other two cell lines were near triploid (HT29, 67-74; SKCO1, 70-77). However, some cells, especially from HT29 and SKCO1, contained much higher numbers of chromosomes per metaphase cell than the ranges indicated in Table 11.

B. Selected Hybrids

Differential G-11 analysis of ten hybrid HT29xRAG subclones indicated that these hybrids contained variable numbers of human chromosomes. This result was found, not only from subclone to subclone, but within each subclone (Table 20). That is, each metaphase cell within a specific subclone contained variable numbers of human chromosomes. For example, subclone F2.131.D2C4 contained a range of human chromosomes from

7 to 18. This property may be a reflection of the actual hybrid cell types or a characteristic property of interspecific hybrids between aneuploid parent cell lines.

Giemsa banding of selected hybrids was undertaken to ascertain whether identification of specific human chromosomes would be possible. Based on the G-11 results there were approximately 18-35 human chromosomes present in the HT29xRAG subclone, F2.179.A9C6. However, Giemsa banding identified only 10 human chromosomes with 3 extra ones that were of uncertain origin (Plate 8). It is evident that identification of human chromosome content is difficult when G-banding is used as the only chromosome identification method. A complementary approach to identifying human chromosomes is isoenzyme analysis of the hybrids particularly as enzyme markers have been defined for each human chromosome. In addition, other chromosome banding methods such as quinacrine banding could be used to confirm the Giemsa banding results.

In retrospect, a few disadvantages have surfaced which have made the characterization of the somatic cell hybrids, produced in the present study, more difficult. First, the human cell line, HT29, failed to show demonstrable secretion of CEA into the supernatant medium. Other investigators have reported that HT29 secretes CEA into growth medium (Egan and Todd, 1972; Shi et al., 1983) but the use of polyclonal anti-CEA antibodies in the assay systems used to measure CEA may explain the difference between their work and the results reported here. Second, the two cell lines (SKCO1 and HT29), which gave the only interspecific hybrids, both have high chromosome numbers (Table 11, with marker

chromosomes (Plates 1 and 3). Both of these factors made identification of human chromosome content in the hybrids difficult. Ideally, the LS174T cell line would have made the best fusion partner with respect to chromosome identification because it had a relatively stable chromosome number (45, X0) and no marker chromosomes.

In the four hybrid cell types produced (SKCO1xRAG, SKCO1xSTO, HT29xRAG and HT29xSTO) none of the hybrids expressed or produced significant levels of CEA, possibly because the CEA gene(s) or genes responsible for the regulation of them may have been suppressed. If CEA is being produced, then it is borderline or below the sensitivity of the assays used for detecting it. Recent work clearly suggests that the RAG cell line may not permit the expression of human-specific antigens (Rettig et al., 1986b; See also Section 4.3.3).

4.3 Other Gene Mapping Studies

4.3.1 Human Tumor-Associated Markers

The somatic cell genetic approach has been successfully used for mapping human tumor associated antigens. For example, the human melanoma-associated antigen p97, a 97 KD cell surface glycoprotein, was mapped by analyzing human fibroblast-mouse melanoma hybrids with highly sensitive and specific immunoassays for p97 antigen and correlating antigen expression with human chromosome 3 (Plowman, Brown, Enns, Schroder, Nikinmaa, Sussman, Hellstrom and Hellstrom, 1983). Another

human cellular tumor antigen called p53 was mapped to the short arm of chromosome 17. This result was based upon isolation of a human p53 cDNA clone, which was used to probe DNA from human fibroblast-rodent (mouse, A9 and B82 lines; Chinese hamster line) somatic cell hybrids after human chromosome segregation. Further analysis by in situ hybridization confirmed this gene assignment (McBride, Merry and Givol, 1986).

4.3.2 Differentiation-Associated Markers

Differentiation-associated antigens have also been mapped using human-rodent somatic cell hybrids. For example, two T cell differentiation antigens, one being a 120 KD antigen (Tp120) and the other being the T4 antigen, a 62 KD glycoprotein associated with the T helper/inducer cells, have been mapped. The Tp120 antigen was mapped to chromosome 11 (Tsuge, Utsumi, Ueda, Takamoto and Takahashi, 1985) and the T4 antigen was mapped to chromosome 12 (Kozbor et al., 1986).

4.3.3 The Permissive versus Nonpermissive Principle

One group of investigators have assigned several antigenic systems to specific human chromosomes using: (1) the immunoassay called "immune rosetting" on viable hybrid cells to determine antigen expression and (2) karyotype (Giemsa banding, G-11) and isoenzyme analysis to determine human chromosome content. During these studies the concept of permissive/nonpermissive rodent cell lines developed to describe the expression of individual antigenic systems. For example, the human nerve growth factor receptor (NGFR) was mapped to chromosome 17 by studying receptor

expression on various human-rodent somatic cell hybrids. However, although chromosome 17 was identified as the chromosome containing the NGFR genes, NGFR was not always expressed in hybrids containing that chromosome. Chromosome 17+ hybrids, for example, derived from fusions between NGFR+ human cells and mouse neuroblastoma cells or mouse cell fibroblasts expressed NGFR. However, 17+ hybrids derived from RAG mouse kidney adenocarcinoma cells did not express NGFR (Rettig et al., 1986b). For this reason RAG was classified as a nonpermissive cell line and the other mouse cell lines were classified as permissive.

4.4 Molecular Genetic Studies of CEA

Molecular genetic approaches in mapping the CEA gene(s) have recently been reviewed (Shively and Beatty, 1985). In this review several cloning strategies were outlined including: (1) isolation of CEA polysomal mRNA with monoclonal anti-CEA antibodies. Because polysomes, the protein synthesizing machinery, contain both CEA protein and CEA mRNA this method can be used to isolate and purify mRNA specific for CEA. The enriched fraction can be used to probe cDNA libraries for potential positive CEA cDNA clones. (2) production of a mixture of synthetic oligonucleotides corresponding to the known NH_2 terminal amino acid sequence can be used in a similar manner as the enriched mRNA fraction to screen for potential positive CEA cDNA clones. Once CEA cDNA is identified it can be used for many genetic studies. Some possible

studies include sequencing the polynucleotide and from that deducing the amino acid sequence, studying the expression of the CEA gene(s) in normal and malignant tissues, and gene transfer and gene mutation studies which may provide insights into the biological function of CEA and the related gene products such as the CEA cross-reactive antigens.

In 1983, Zimmermann reported isolation and purification of the messenger RNA that codes for the CEA precursor. Following translation into radiolabelled proteins, polyclonal and monoclonal anti-CEA antibodies were used to select CEA protein. Analysis on SDS-PAGE demonstrated a protein with an apparent molecular weight of about 85 KD. Taking into account the high carbohydrate content of CEA (approx. 60%) the observed molecular weight of the CEA precursor protein is in good agreement with the total molecular weight of 180 KD reported for native CEA (Zimmermann, Friedrich, Grunert, Luckenbach, Thompson and von Kleist, 1983). This has been complemented by the report that three CEA-producing cell lines contain CEA unglycosylated protein ranging in molecular weight from 78 to 83 KD. Protein backbones of CEA were identified and analyzed by SDS-PAGE (Kuroki, Kuroki, Ichiki and Matsuoka, 1984).

DNA mediated gene transfer (DMGT) has been demonstrated to be a potentially useful technique for characterizing the structural genes for CEA. DNA from the CEA-producing cell line, HCT-8R, was used to transfect mouse recipient cells by the calcium precipitation method. Using monoclonal anti-CEA antibodies to screen the transfected clones, the identification of two CEA producing clones resulted. Further analysis with SDS-PAGE demonstrated membrane associated moieties which co-migrated

with purified CEA (Fuks, Price, Stanners and Gold, 1983).

In addition, a cDNA library has been constructed using poly A+ mRNA purified from LS174T. Using nucleotide probes corresponding to the NH_2-terminal sequence of CEA several positive clones were identified. Analysis of translation proteins from the LS174T poly A+ mRNA, using polyclonal anti-CEA antibodies, revealed a protein with an apparent molecular weight of approximately 70 KD (noor Khan, Castro, Zoubir, Gunne, Hammarstrom, Lee, Lake and Heden, 1985).

Very recently, a group reported the identification of several CEA positive recombinant DNA clones (Oikawa, Nakazato and Kosaki, 1987). DNA sequencing of these positive clones has revealed both the actual length of the protein moiety (668 amino acids) and the amino acid sequence. Another group has confirmed some of the internal amino acid sequencing using an improved method for analyzing peptide sequences in highly glycosylated proteins (Paxton, Mooser, Pande, Lee and Shively, 1987). From the amino acid sequencing data for CEA, this group have postulated that CEA may be a member of the immunoglobulin supergene family.

4.6 Conclusions and Future Considerations

The primary objective of this study was to produce somatic cell hybrids between CEA expressing/secreting colonic carcinoma cell lines and mouse cell lines and that was achieved with SKCO1, HT29, RAG and STO. However, CEA levels in these hybrids, in terms of membrane and

cytoplasmic expression or in terms of secretion, was borderline and no hybrids with higher, more definite, CEA expression/secretion were obtained. The major problem identified was the lack of success in producing hybrids which would express the tumor marker in significant quantities.

CEA expression/production appears to be repressed or lowered significantly by fusion with the mouse cell lines RAG and STO. Mouse cell lines, which have been fused with human cell lines for the study of tissue-restricted cell markers and result in repression of the marker of interest, have been classified as "nonpermissive" cell lines. RAG has already been labelled as nonpermissive for some differentiation specific antigens and it may also be appropriate to classify the cell line STO as nonpermissive for the expression of the CEA gene(s).

In view of the low expression/secretion values for the HT29xRAG, SKCO1xSTO and the SKCO1xRAG hybrids an avenue which should be explored as a follow-up to this study is fusion of SKCO1 (the highest CEA expressor/secretor in our hands) with the A9 fibroblast line which has been defined as a permissive/inducive cell line (Rettig et al., 1986a). The results in this thesis have shown that a successful fusion procedure is available for such a monolayer x monolayer fusion and all the methods of analysis (immunological and chromosomal) have been worked out. If definite CEA producing hybrids were obtained but chromosomal analysis (because of the SKCO1 parental cell line) was difficult, then complementary isoenzyme analysis could be used to confirm human chromosome content.

Consideration could also be given to using LS174T again, but with higher

selection concentrations of ouabain.

Development of the "right" CEA expressing hybrids between a high CEA producing parent cell line and a permissive/inducing mouse cell line would be invaluable in future studies. Clearly, the somatic cell genetic approach will be complementary to other molecular biological approaches also being investigated at present for the mapping of the CEA gene(s).

Finally, a possible use for the interspecific hybrids developed in this project is for mapping other differentiation specific antigens. For example, the LS174TxRAG hybrids produced by Sheer, while of no value for mapping the CEA gene(s), have been used to map the epithelium-specific AUAI antigen to chromosome 2 (Spurr, Durbin, Sheer, Parkar, Bobrow and Bodmer, 1986).

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Appendix I

1. Fusion Coding System

F1 to Fn refers to specific fusion number

F1.(1 to 192) refers to the number of possible hybrids if 100% of the 2x96 well culture plates contain hybrid colonies.

F1.1 (A1 to A12)
(B1 to B12)
(C1 to C12)
(D1 to D12)
(E1 to E12)
(F1 to F12)
(G1 to G12)
(H1 to H12)

refers to the specific hybrid clone (for eg. F1.1.C5 is the hybrid clone isolated from well C5 in a 96 well culture plate. The fusion number is one and the original hybrid is one).

F1.1.A1 (A1 to A12)
(B1 to B12)
(C1 to C12)
(D1 to D12)
(E1 to E12)
(F1 to F12)
(G1 to G12)
(H1 to H12)

refers to the specific hybrid subclone obtained by limiting dilution from F1.1.A1 hybrid clone.

Table 1. Original hybrid colonies selected for cloning.

Fusion Code	Parent Cell Lines	Original Hybrid Colonies Selected for Cloning	
F2	HT29	F2.19	F2.140
	RAG	F2.66	F2.144
		F2.67	F2.150
		F2.70	F2.158
		F2.116	F2.160
		F2.121	F2.161
		F2.130	F2.169
		F2.131	F2.178
		F2.133	F2.179
F3	SKCO1	F3.2	
	STO	F3.3	
F4	HT29	F4.52	F4.92
	STO	F4.56	F4.98
		F4.57	F4.108
		F4.86	F4.119
		F4.90	
F5	SKCO1	F5.2	
	RAG		

Table 2. Hybrid clones obtained by limiting dilution.

Primary Hybrid	No. Clones Obtained	Primary Hybrid	No. Clones Obtained
F2.19	12	F2.160	9
F2.66	4	F2.161	6
F2.67	4	F2.169	8
F2.70	5	F2.178	9
F2.116	3	F2.179	15
F2.121	1	F3.2	0
F2.130	11	F3.3	2
F2.131	13	F4.52	1
F2.133	3	F4.56	1
F2.140	8	F4.86	6
F2.144	2	F4.90	0
F2.150	1	F5.2	4
F2.158	3		

Total Clones obtained = 131

Table 3 Primary hybrid clones selected for re-cloning.

Fusion Code	Parent Cell Lines	Primary Hybrid Clones Selected for Re-cloning	
F2	HT29	F2.19.C11	F2.144.F9
	RAG	F2.19.D4	F2.160.B8
		F2.121.B4	F2.169.E9
		F2.130.F9	F2.169.F5
		F2.131.D2	F2.178.C7
		F2.131.F10	F2.179.A9
		F2.131.H1	F2.179.B7
		F2.140.A7	F2.179.E10

16 out of 20

Note: There were no primary hybrids selected from F3, F4 or F5 for re-cloning.

Table 4. Hybrid subclones obtained by limiting dilution.

Secondary Clone	No. Subclones Obtained
F2.169.E9	14
F2.19.C11	8
F2.131.D2	21
F2.179.B7	20
F2.121.B4	1
F2.140.A7	5
F2.130.F9	13
F2.179.A9	11
F2.131.F10	14
F2.19.D4	3
F2.169.F5	4
F2.178.C7	4
F2.160.B8	4
F2.144.F9	4
F2.131.H1	0
F2.179.E10	0
Total Subclones = 126	

Table 5. Secondary clones selected for stringent CEA analysis.

Fusion Code	Parent Cell Lines	Secondary Clones Selected for Further CEA Analysis	
F2	HT29	F2.130.F9D2	F2.169.E9F1
	RAG	F2.131.D2C4	F2.169.E9F7
		F2.131.D2F2	F2.179.A9C6
		F2.169.E9E1	F2.179.B7D8
		F2.169.E9E4	F2.179.B7E8
			10 out of 104

Appendix II

Notes for the interpretation of ELISA data.

1. Individual assay results can only be compared to controls for that day. i.e. comparison of absolute absorbance values between assays is invalid. For example, differences in antibody batches and reagents may result in variations in absorbances between assays.

2. The substrate incubation time was varied. Initially, it was 15-20 min. but in an attempt to maximize the chances of detecting a CEA positive hybrid the time was increased to 40-60 min.

A. LS174T Ouabain Resistance - ELISA Data

I. ELISA #1.

Incubation time with substrate 20 min.

		Absorbance
Controls	1. CEA (100 ng per ml)	1.164
	2. RPMI-HAT medium	0.269
	3. 1% BSA/PBS/Tween	0.393

Sample No.	Absorbance	Sample No.	Absorbance
1	0.347	7	0.931
2	0.783	8	0.611
3	0.515	9	0.564
4	0.704	10	0.581
5	0.597	11	0.706
6	0.467	12	2.770

II. ELISA #2

Incubation time with substrate addition 20 min.

		Absorbance
Controls:	1. CEA (100 ng per ml)	1.915
	2. RPMI-HAT medium	0.105
	3. 1%BSA/PBS/Tween	0.098

Sample No.	Absorbance	Sample No.	Absorbance
13	0.467	30	0.380
14	0.437	31	0.390
15	0.503	32	0.338
16	0.147	33	0.193

cont'd

Sample No.	Absorbance	Sample No.	Absorbance
17	0.428	34	0.136
18	0.252	35	0.342
19	0.594	36	0.435
20	0.263	37	1.523
21	0.136	38	0.249
22	0.537	39	0.343
23	0.114	40	0.369
24	0.064	41	0.661
25	0.414	42	0.482
26	0.329	43	0.361
27	0.161	44	0.147
28	0.082	45	0.566
29	0.404	46	0.209

III. ELISA #3

Incubation time with substrate 20 min.

	Absorbance
Controls: 1. CEA (100 ng per ml)	1.833
2. RPMI-HAT medium	0.091
3. 1%BSA/PBS/Tween	0.054

Sample No.	Absorbance	Sample No.	Absorbance
47	0.614	60	1.197
48	0.627	61	1.221
49	0.006	62	1.242
50	0.025	63	0.889
51	0.556	64	0.970
52	0.989	65	0.698
53	0.361	66	1.017
54	1.458	67	1.255
55	0.639	68	1.427
56	0.330	69	1.325
57	0.564	70	1.322
58	0.109	71	1.153
59	0.033	72	1.203

NOTE: The above samples tested in ELISAs 1, 2 and 3 were all derived from fusions between the human parent cell line, LS174T, and the mouse cell lines NS1 or SP2/0. Because these samples were determined to be ouabain resistant LS174T cells they were not coded using the fusion coding system.

B. HT29xNS1 - ELISA Data

IV. ELISA #4

Incubation time with substrate 20 min.

Controls:		Absorbance
1.	CEA (100 ng per ml)	2.897
2.	CEA (3 ng per ml)	0.305
3.	1% BSA/PBS/Tween	0.216

Sample No.	Absorbance
1	0.309

V. ELISA #5

Incubation time with substrate 15 min.

Controls:		Absorbance
1.	CEA (100 ng per ml)	2.685
2.	CEA (3 ng per ml)	0.323
3.	1% BSA/PBS/Tween	0.318

Sample No.	Absorbance
1	0.331

C. ELISA Data - HT29xRAG, HT29xST0, SKC01xRAG and SKC01xST0

VI. ELISA #6

Incubation time with substrate 60 min.

	Absorbance
Controls: 1. CEA (100 ng per ml)	>3.2
2. CEA (3 ng per ml)	1.941
3. RPMI-HAT medium	1.561
3. 1%BSA/PBS/Tween	1.380

Samples:

Fusion Code No.	Absorbance	Fusion Code No.	Absorbance
F2.1	1.194	F2.39	1.651
F2.2	1.158	F2.40	1.586
F2.3	1.091	F2.41	1.600
F2.4	0.939	F2.42	1.681
F2.5	1.338	F2.43	1.415
F2.6	1.529	F2.44	1.417
F2.7	1.541	F2.45	1.807
F2.8	1.106	F2.46	1.899
F2.9	1.377	F2.47	1.940
F2.10	1.579	F2.48	1.708
F2.11	1.449	F2.49	1.650
F2.12	1.467	F2.50	1.600
F2.13	1.397	F2.51	1.532
F2.14	1.149	F2.52	1.663
F2.16	1.830	F2.54	1.240
F2.17	1.925	F2.55	1.503
F2.18	1.566	F2.56	1.846
F2.19	2.024	F2.57	1.728
F2.21	1.495	F2.59	1.765
F2.22	1.642	F2.60	1.859
F2.23	1.330	F2.61	1.605
F2.24	1.459	F2.62	1.898
F2.25	1.642	F2.63	1.392
F2.26	1.718	F2.64	1.290
F2.27	1.646	F2.65	1.925
F2.28	1.430	F2.66	2.076
F2.29	1.355	F2.67	2.220
F2.30	1.685	F2.68	1.457
F2.31	1.327	F2.69	1.493
F2.32	1.572	F2.70	2.164
F2.33	1.525	F2.71	1.507
F2.34	1.251	F2.72	1.731
F2.35	1.798	F2.73	1.289
F2.36	1.827	F2.74	1.228
F2.37	1.650	F2.75	1.293
F2.15	1.504	F2.20	1.546

cont'd

Fusion Code No.	Absorbance	Fusion Code No.	Absorbance
F2.38	1.697	F2.76	1.442
F2.77	1.579	F2.120	1.914
F2.78	1.321	F2.121	2.044
F2.79	1.388	F2.122	1.611
F2.80	1.617	F2.123	1.627
F2.81	1.258	F2.124	1.841
F2.83	1.216	F2.126	1.637
F2.84	1.289	F2.127	1.253
F2.85	0.971	F2.128	1.291
F2.86	1.391	F2.129	1.795
F2.87	1.710	F2.130	2.006
F2.88	1.586	F2.131	2.148
F2.89	1.519	F2.132	1.272
F2.90	1.392	F2.133	2.406
F2.91	1.493	F2.134	1.582
F2.92	1.631	F2.135	1.543
F2.93	1.483	F2.136	1.647
F2.94	1.295	F2.137	1.878
F2.95	1.031	F2.138	1.418
F2.96	1.314	F2.139	1.801
F2.97	1.795	F2.140	2.050
F2.98	1.724	F2.141	1.834
F2.99	1.377	F2.142	1.343
F2.100	1.743	F2.143	1.748
F2.102	1.759	F2.145	1.471
F2.103	1.704	F2.146	1.921
F2.105	1.796	F2.148	1.282
F2.106	1.386	F2.149	1.789
F2.108	1.098	F2.151	1.932
F2.109	1.802	F2.152	1.481
F2.110	1.837	F2.153	1.667
F2.111	1.903	F2.154	1.931
F2.112	1.966	F2.155	1.401
F2.113	1.734	F2.156	1.670
F2.115	1.791	F2.184	1.254
F2.116	1.974	F2.185	1.264
F2.117	1.530	F2.144	1.970
F2.118	1.367	F2.147	1.575
F2.119	1.896	F2.150	2.157
F2.114	1.791	F2.125	1.468

cont'd

Fusion Code No.	Absorbance	Fusion Code No.	Absorbance
F3.1	1.475		
F3.2	1.829		
F3.3	1.874		
F4.39	1.255	F4.50	1.296
F4.40	1.105	F4.55	1.201
F4.41	1.179	F4.58	1.252
F4.42	1.177		
F4.47	1.087		
F4.49	1.182		
F5.6	1.055	F5.14	0.910
F5.7	1.246	F5.15	0.960
F5.8	1.055	F5.16	1.094
F5.9	1.064	F5.17	1.288
F5.10	1.004	F5.18	1.198
F5.11	1.005	F5.19	1.084
F5.12	1.053	F5.20	1.252
F5.13	1.015		

VII. ELISA #7 *

Incubation time with substrate 14 hrs

	Absorbance
Controls: 1. CEA (12 ng per ml)	0.247
2. CEA (3 ng per ml)	0.194
3. 1%BSA/PBS/Tween	0.192

Samples:

Fusion Code No.	Absorbance	Fusion Code No.	Absorbance
F4.108	0.247	F2.182	0.182
F4.109	0.230	F2.183	0.185
F4.115	0.234	F2.181	0.186
F4.119	0.246	F2.180	0.194
F4.52	0.289	F2.179	0.262
F4.56	0.303	F2.178	0.210
F4.124	0.199	F2.177	0.179
F4.122	0.175	F2.176	0.177
F4.120	0.198	F2.175	0.177
F4.63	0.182	F2.174	0.169
F4.66	0.188	F2.173	0.188
F4.103	0.183	F2.172	0.183
F4.128	0.224	F2.167	0.201
F4.57	0.379	F2.168	0.209
F4.54	0.196	F2.169	0.234
F4.43	0.193	F2.170	0.188
F4.27	0.180	F2.171	0.195
F4.67	0.194	F2.166	0.210
F4.45	0.181	F2.165	0.208
F4.46	0.172	F2.164	0.197
F4.53	0.182	F2.162	0.213
F4.60	0.170	F2.157	0.219
F4.44	0.179	F2.158	0.272
F4.37	0.186	F2.159	0.219
F4.31	0.208	F2.160	0.238
F4.28	0.179	F2.161	0.236
F4.24	0.225	F2.163	0.207
F4.97	0.217	F5.21	0.207
F4.92	0.227	F5.24	0.209
F4.90	0.235	F5.2	0.233
F4.86	0.240	F5.23	0.176
F4.98	0.244	F5.1	0.181

*Overnight incubation; assay performed in a microplate and not cuvettes.

VIII. ELISA #8

Incubation time with substrate 60 min.

		Absorbance
Controls:	1. CEA (12 ng per ml)	1.363
	2. CEA (3 ng per ml)	0.875
	3. HAT-RPMI medium	0.655
	4. 1%BSA/PBS/Tween	0.685

Samples:

Fusion Code No.	Absorbance	Fusion Code No.	Absorbance
F2.133.H6	0.674	F2.70.H7	0.585
F2.144.F2	0.626	F2.66.D8	0.650
F2.121.B4	0.772	F2.140.A7	0.764
F2.116.C10	0.613	F2.161.A4	0.634
F2.116.D6	0.564	F2.160.B12	0.693
F2.131.B6	0.567	F2.160.F6	0.621
F2.131.C2	0.636	F2.179.C8	0.509
F2.131.C8	0.575	F2.179.F7	0.534
F2.131.C11	0.543	F2.179.H5	0.674
F2.131.E2	0.661	F2.67.E2	0.604
F2.131.E12	0.640	F2.131.B8	0.623
F2.131.E5	0.600	F2.131.D2	0.727
F2.131.H1	0.713	F2.131.D8	0.654
F2.178.B10	0.681	F2.131.F10	0.700
F2.140.E9	0.642	F2.70.D11	0.656
F2.67.D8	0.575	F2.130.F9	0.801
F2.66.E8	0.699	F2.67.C3	0.644
F2.130.F4	0.639	F2.140.H10	0.673
F2.133.A6	0.695	F2.179.A9	0.803
F2.150.C5	0.699	F2.179.B7	0.706
F2.169.F5	0.722	F2.179.F9	0.651
F2.169.E9	0.720	F2.160.G8	0.572
F2.169.E3	0.620		
F2.169.B10	0.569	F5.2.E7	0.594
F2.169.A9	0.561	F5.2.E4	0.550
F2.169.A8	0.659	F5.2.B5	0.633
F2.160.G4	0.672		
F2.178.D7	0.618		
F2.178.D5	0.696		
F2.178.C7	0.728		
F2.169.H8	0.635		
F2.179.C10	0.690		
F2.133.A3	0.485		
F2.130.G11	0.565		
F2.70.B8	0.657		
F2.160.B8	0.710		

IX. ELISA #9

Incubation time with substrate 60 min.

Controls:		Absorbance
1.	CEA (12 ng per ml)	1.397
2.	CEA (3 ng per ml)	1.153
3.	HAT-RPMI medium	1.044
3.	1%BSA/PBS/Tween	0.894

Samples:

Fusion Code No.	Absorbance	Fusion Code No.	Absorbance
F2.116.B11	0.945	F2.179.F8	0.964
F2.130.A5	1.007	F2.130.E8	0.927
F2.66.D12	1.043	F2.160.D11	0.889
F2.66.F6	0.800	F2.70.C5	0.896
F2.160.E7	0.746	F2.178.E8	0.893
F2.140.E1	0.751	F2.178.E9	0.715
F2.140.D10	0.708	F2.130.E3	0.880
F2.158.E4	0.724	F2.130.C9	0.892
F2.169.B3	1.113	F2.130.B10	1.110
F2.179.B8	1.020	F2.130.B9	1.001
F2.179.C7	1.129	F2.131.C1	0.984
F2.179.D9	0.879	F2.130.E4	1.002
F2.179.F6	0.868	F2.130.E11	0.892
F2.179.H2	0.865	F2.178.B7	0.770
F2.179.H11	1.016	F2.178.F10	0.719
F2.70.C7	0.860	F2.178.H3	0.762
F2.160.D7	1.056	F2.161.E10	0.980
F2.67.F10	1.127	F2.161.B9	0.994
F2.179.E10	1.231	F5.2.E2	0.892

X. ELISA #10

Incubation time with substrate 45 min.

		Absorbance
Controls:	1. CEA (12 ng per ml)	1.163
	2. CEA (3 ng per ml)	0.877
	3. 1%BSA/PBS/Tween	0.698

Samples:

Fusion Code No.	Absorbance	Fusion Code No.	Absorbance
F2.169.E9B8	0.841	F2.179.B7A5	0.738
F2.169.E9B10	0.754	F2.179.B7A10	0.810
F2.169.E9C8	0.725	F2.179.B7B4	0.769
F2.169.E9E1	0.932*	F2.179.B7B10	0.710
F2.169.E9E4	0.852*	F2.179.B7C8	0.747
F2.169.E9F1	0.921*	F2.179.B7D8	0.869*
F2.169.E9F7	0.850*	F2.179.B7D10	0.834
F2.169.E9H9	0.802	F2.179.B7E10	0.773
F2.179.B7E12	0.786	F2.179.B7E11	0.684
F2.161.E4	0.827		
F2.19.B4	0.706		
F2.19.B8	0.721		
F2.19.C5	0.904		
F2.19.C9	0.847		
F2.19.C11	0.918		
F2.19.D3	0.767		
F2.19.D4	0.879		
F2.19.E2	0.698		
F2.19.G7	0.717		
F2.161.C9	0.825		
F2.160.B10	0.747		
F2.161.E6	0.744		
F2.158.B4	0.680		
F2.140.G7	0.682		
F2.140.D7	0.834		
F2.140.C8	0.823		
F2.158.B8	0.823		

* The five subclones with the highest absorbance in this ELISA were selected for further evaluation.

XI. ELISA #11

Incubation time with substrate 45 min.

		Absorbance
Controls:	1. CEA (12 ng per ml)	0.654
	2. CEA (3 ng per ml)	0.343
	3. HAT-RPMI medium	0.526
	4. 1%BSA/PBS/Tween	0.452

Samples:

Fusion Code No.	Absorbance	Fusion Code No.	Absorbance
F2.169.E9G6	0.677	F2.179.B7F7	0.490
F2.179.B7A8	0.605	F2.179.B7F9	0.538
F2.179.B7D4	0.592	F2.131.D2A4	0.480
F2.179.B7F1	0.613	F2.131.D2A7	0.537
F2.179.B7F6	0.467	F2.131.D2B11	0.425
F2.131.F10H7	0.405	F2.131.D2E1	0.434
F2.169.F5E8	0.601	F2.131.D2D11	0.485
F2.179.A9C4	0.526	F2.131.D2D5	0.564
F2.179.A9D10	0.441	F2.131.D2D4	0.449
F2.179.A9G3	0.452	F2.131.D2C10	0.502
F2.179.A9F9	0.478	F2.131.D2E3	0.560
F2.179.A9F3	0.592	F2.131.D2E10	0.486
F2.179.A9E10	0.332	F2.131.D2E12	0.522
F2.179.A9E9	0.527	F2.131.D2H2	0.473
F2.179.A9G5	0.549	F2.131.D2H4	0.486
F2.179.A9G7	0.561	F2.140.A7G4	0.464
F2.130.F9C9	0.645	F2.140.A7E12	0.479
F2.130.F9D2	0.697	F2.140.A7B4	0.561
F2.131.F10C7	0.677	F2.131.D2H9	0.528
F2.131.D2F2	0.704	F2.131.D2H8	0.515
F2.131.D2C4	0.497	F2.178.C7C9	0.644
F2.140.A7D7	0.664	F2.178.C7D11	0.551
F2.169.E9C7	0.585	F2.178.C7E8	0.521
F2.131.F10G8	0.589	F2.169.E9D4	0.589
F2.131.D2F5	0.724	F2.169.E9E6	0.594
F2.131.D2F7	0.614	F2.130.F9C7	0.485
F2.179.B7B7	0.613	F2.130.F9B9	0.510
F2.160.B8B10	0.585	F2.130.F9B8	0.597
F2.160.B8D3	0.632	F2.130.F9B2	0.419
F2.178.C7G10	0.546	F2.169.E9F8	0.480
F2.131.D2G5	0.479	F2.130.F9D8	0.597
F2.144.F9D8	0.348	F2.130.F9F4	0.548
F2.144.F9C12	0.569	F2.130.F9F11	0.495
F2.144.F9B7	0.523	F2.130.F9G5	0.529
F2.179.B7E4	0.392	F2.130.F9G7	0.510
F2.131.D2G7	0.493	F2.131.F10F1	0.551

cont'd

Fusion Code No.	Absorbance	Fusion Code No.	Absorbance
F2.131.F10E9	0.507	F2.19.F9	0.497
F2.131.F10C3	0.581	F2.19.E9	0.520
F2.131.F10B4	0.393	F2.144.F9	0.771
F2.131.F10B2	0.440	F2.19.G10	0.597
F2.131.F10E8	0.536	F3.3.D2	0.622
F2.131.F10F8	0.560		
F2.169.E9B6	0.550		
F2.131.F10G7	0.508		
F2.169.F5E4	0.535		
F2.169.F5A7	0.499		
F2.169.F5F10	0.489		
F2.131.F10H8	0.442		
F2.179.A9C6	0.895		
F2.130.F9E8	0.561		
F2.131.F10D11	0.628		
F2.179.B7E8	0.688		

XII. ELISA #12

Incubation time with substrate 60 min.

	Absorbance
Controls: 1. CEA (12 ng per ml)	1.246
2. CEA (3 ng per ml)	0.781
3. HAT-RPMI medium	0.774
4. 1%BSA/PBS/Tween	0.873

Samples:

Fusion Code No.	Absorbance	Fusion Code No.	Absorbance
F2.19.C11E10	0.708	F4.86.F11	0.729
F2.130.F9B11	0.718	F4.86.B3	0.701
F2.131.F10C8	0.728	F4.86.C3	0.679
F2.179.A9E4	0.817	F4.86.E7	0.635
F2.144.F9F9	0.935	F4.86.E5	0.887
F2.160.B8C10	0.907	F4.52.B2	0.853
F2.140.A7B5	0.957	F4.86.H9	0.554
F2.160.B8F9	0.859		
F2.19.D4D1	0.920	F3.3.F9	0.764
F2.19.C11H9	1.166		
F2.19.D4C1	1.206		
F2.19.D4B7	0.962		
F2.19.C11F7	0.921		
F2.19.C11G5	0.658		
F2.19.C11D4	1.026		
F2.19.C11C5	1.030		
F2.19.C11A2	0.939		

XIII. ELISA #13

Incubation time with substrate 15 min.

		Absorbance
Controls:	1. CEA (100 ng per ml)	2.070
	2. CEA (3 ng per ml)	0.656
	3. HAT-RPMI medium	0.749
	4. 1%BSA/PBS/Tween	0.399

Samples:

Fusion Code No.	Absorbance
F2.19.C11E4	0.828
F2.121.B4F7	0.785
F2.179.B7F11	0.833
F4.56.D8	0.706

XIV. ELISA #14

Incubation time with substrate 40 min.

	Absorbance
Controls: 1. CEA (50 ng per ml)	0.393
2. CEA (3 ng per ml)	0.109
3. RPMI-HAT medium	0.100
4. 1% BSA/PBS/Tween	0.171

Samples:

	Absorbance
A. HT29 (Day 2)	0.193
HT29 (Day 4)	0.138
HT29 (Day 6)	0.136
HT29 (Day 8)	0.171
HT29 (Day 10)	0.177
HT29 (Day 12)	0.176
HT29 (Day 14)	0.201
HT29 (Day 16)	0.176
HT29 (Day 18)	0.184
HT29 (Day 20)	0.122
HT29 (Day 22)	0.149

	Absorbance (Day 6 samples)	Absorbance (Day 9 samples)
B. HT29xRAG subclones		
F2.169.E9E1	0.229	0.121
F2.169.E9F1	0.176	0.168
F2.169.E9E4	0.159	0.161
F2.169.E9F7	0.167	0.179
F2.130.F9D2	0.163	0.153
F2.131.D2F2	0.168	0.118
F2.131.D2C4	0.161	0.178
F2.179.B7D8	0.062	0.008
F2.179.B7E8	0.156	0.155
F2.179.A9C6	0.121	0.050
C. SKC01xRAG primary hybrids		
F5.2	0.190	0.130
F5.14	0.119	0.119
F5.16	0.189	0.113
F5.19	0.111	0.128
D. SKC01xSTO primary hybrids		
F3.2	0.162	0.173
F3.3	0.154	0.111
E. Mouse parent cell lines		
RAG	0.206	
STO	0.166	

XV. ELISA #15

Incubation time with substrate 40 min.

		Absorbance
Controls:	1. CEA (100 ng per ml)	2.805
	2. CEA (3 ng per ml)	0.568
	3. RPMI-HAT	0.338
	4. 1%BSA/PBS/Tween	0.332

Samples:

A. Spent medium (Day 6 and Day cultures)

Fusion Code No.	Absorbance (Day 6)	Absorbance (Day 9)
F5.4	0.263	0.247
F5.3	0.266	0.285
F5.6	0.303	0.251
F5.10	0.330	0.326
F5.11	0.367	0.243
F5.12	0.329	-
F5.13	0.263	0.266
F5.15	0.289	0.314
F5.17	0.295	0.330
F5.20	0.268	0.275
F5.21	0.326	0.398

B. Concentrated Spent medium

Fusion Code No.	Absorbance
F5.6	0.328
F5.17	0.205
F5.2	0.162
F5.19	0.293
F2.179.A9C6	0.295
F2.131.D2C4	0.309
F3.2	0.337
F3.3	0.387
RPMI-HAT	0.319

XVI. ELISA #16

Incubation time with substrate 20 min.

		Absorbance
Controls:	1. CEA (100 ng per ml)	2.145
	2. CEA (75 ng per ml)	2.083
	3. CEA (50 ng per ml)	1.320
	4. CEA (25 ng per ml)	0.739
	5. CEA (12 ng per ml)	0.649
	6. CEA (6 ng per ml)	0.501
	7. CEA (3 ng per ml)	0.432
	8. RPMI-HAT	0.294
	9. 1%BSA/PBS/Tween	0.340

Samples: Cytosol and membrane subcellular fractions

Fusion Code No.	Absorbance (cytosol)	Absorbance (membrane)
F2.179.A9C6	0.543	0.355
F2.131.D2C4	0.469	0.329
F5.2	0.555	0.320
F5.19	0.372	0.486
RAG	0.685	0.356
STO	0.585	0.303