FLOW SORTING IN THE STUDY OF TERATOCARCINOMA CELL DIFFERENTIATION

(FLOW SORTING BIJ HET ONDERZOEK NAAR TERATOCARCINOMA CEL DIFFERENTIATIE)

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CONTENTS p.

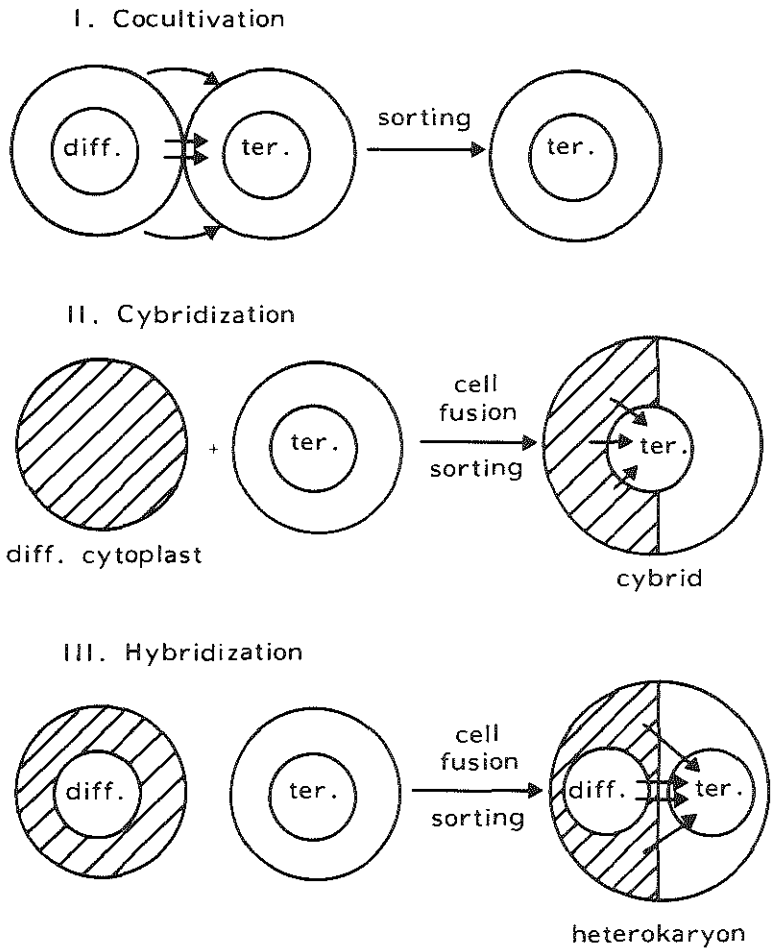

Figure 1 *Three cell culture systems used to search for cellular factors capable of inducing teratocarcinoma cell differentiation. Cellular-, nucleocytoplasmic- and nuclear interactions are indicated by arrows. Flow sorting is used for the selection of cells or fusion products for further culture and analysis. diff. = differentiated (cell), ter. = teratocarcinoma*

General introduction.

Flow cytometry is a technique by which particles (cells, subcellular fragments, bacteria) in aqueous suspension are passed one by one through a sensing region where optical (or electrical) signals are generated. These signals for each individual cell are collected and processed, and may be stored to yield the distribution of the property measured for the population of cells analyzed. Most often cells are fluorescently labelled (with fluorescent dyes, particles or antibodies) and fluorescence signals are measured. Instruments called flow sorters in addition have a sorting capability which allows physical separation of a desired subpopulation for further analysis. This thesis is concerned with the cell biological application of such an instrument with particular emphasis on the use of the sorting capabilities.

The process of cell specialization which occurs when a multicellular organism develops from one cell into the mature organism is called cell differentiation. Mouse teratocarcinoma cells share many properties with early embryonic cells and can be used as an *in vitro* cell model system to study the early events of cell differentiation. Some aspects of teratocarcinoma cell differentiation may especially profitable be studied by making use of the sorting capabilities of the cell sorter and this is what the main part of this thesis is about.

Teratocarcinoma cells can be induced to differentiate by treatment with chemical inducers. Analysis of properties changing upon differentiation can yield information about their role in the differentiation process. One of these properties is the "fluidity" of the plasma membrane. A part of this thesis describes the flow cytophotometric measurement of this change after induction of differentiation.

Three different *in vitro* cell culture systems have been used in this thesis to search for cellular factors capable of inducing teratocarcinoma cell differentiation (figure 1).

I. In a cocultivation system teratocarcinoma cells were cocultivated with differentiated cells to see whether cell-cell interactions could stimulate differentiation. The cell sorter was used to sort the teratocarcinoma cells from the cocultivation mixture for analysis.

II. In a cybridization system teratocarcinoma cells were fused with enucleated differentiated cells (cytoplasts) to see whether exposure of the teratocarcinoma nuclei to the cytoplasm of differentiated cells could induce permanent differentiation. Here the cell sorter was used both for the preparation of the cytoplasts and the selection of the cybrids from the fusion mixture.

III. In a hybridization system teratocarcinoma cells were fused with whole differentiated cells to see whether the teratocarcinoma cells could be induced to produce products of differentiated cells. In this case the cell sorter was used to isolate the heterokaryons from the fusion mixture for analysis.

Chapter 1.

Flow sorting.

1.1 Introduction.

Flow cytometry- the measurement of cells in flow- has evolved during the last 25-30 years. The sheath flow principle (see below) which is used in almost all flow cytometry instruments today was described by Crossland-Taylor in 1953 (1), and the first prototype instruments of flowcytophotometers have been produced around 1967 (2,3,4,5,6). A detailed historical review of the development of flow cytometers and sorters has been published (7).

The technique of flowcytophotometry allows optical measurements on single particles (cells, subcellular fragments, bacteria and so on) flowing one by one through a sensing region. This capacity for single cell measurement is the strength of the technique because it allows analysis of heterogeneous samples. In addition specific subsets of cells from such a sample can be isolated through a coupled possibility for sorting. Since about 3000 cells/s can be handled, sufficient quantities can be isolated in reasonable time for subculture and analysis.

Commercial instruments are available and are being used in a still increasing number of laboratories and this has led to a large number of applications in cell biology and clinical medicine. A number of excellent reviews covering applications in different fields are available (8,9,10,11,12,13,14,15,16,165). In the last section of this chapter applications in cell biology illustrating the use of the sorting capabilities and the various ways cells can be fluorescently labelled, will be discussed in more detail.

Although the principle is the same, different commercial instruments have different properties (17). In the next section the general principle will be elaborated describing the FACS II cell sorter. This is the instrument which was used for the experiments described in this thesis.

1.2 FACS II cell sorter.

A schematic representation showing the basic principle of measurement on the FACS II (Fluorescence Activated Cell Sorter) cell sorter is given in figure 1.1. A sample stream injects cells in a fluid stream which then flows through a small opening in the nozzle forming a fluid jet in air. Inside the nozzle the fluid is in laminary flow and as a result the cells are confined to the center of the fluid stream (sheath-flow principle (1)). Just below the opening in the nozzle a laser beam crosses the fluid jet. Cells passing the laser beam are illuminated one by one and optical signals are generated for each cell. Forward light scatter (roughly proportional

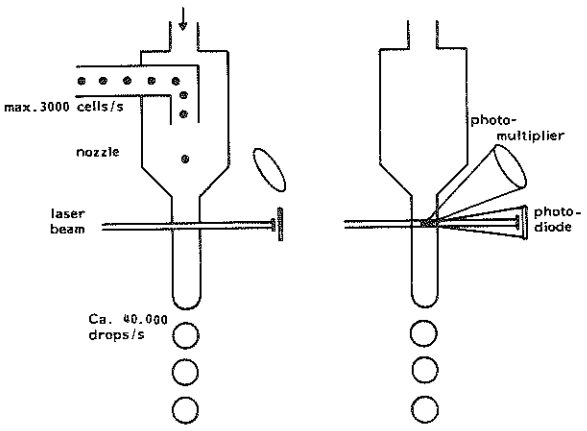

Figure 1.1 Principle of measurement on the FACS II. Cells in a fluid jet, one by one pass a laser beam. Fluorescence and light scatter is generated for each cell and is collected with a photomultiplier and a photodiode.

to cell size) is collected with a photodiode. At 90 degrees a fluorescence signal (or 90° light scatter) can be detected with a photomultiplier. The electrical pulses from these detectors are processed very fast and can be displayed as histograms and dot plots (figure 1.2). Based on this analysis sorting criteria can be specified to select specific subpopulations. Ultrasonic vibration causes breakup of the jet into uniform droplets, which traverse a region of high electric field density. When a cell, satisfying the sorting criteria, has passed the laser beam, the jet is momentarily electrically charged at the moment the cell reaches the end where droplet formation occurs. This droplet is then deflected and collected separate from the main stream (figure 1.3).

To this basic set-up a number of variations and extensions are possible.

- Two colour analysis. If the cells are labelled with two kinds of fluorescent molecules, emitting different colours of fluorescence, these two colours can be detected separately with two photomultipliers.
- Polarization analysis. Using two photomultipliers, two polarization directions can be measured in order to determine the degree of polarization of the emitted fluorescent light.
- Two laser excitation. Two laser beams, one just below the other, can be used to excitate two types of fluorescent molecules at their own optimum excitation wavelenghts.
- Single cell sorting. Instead of sorting all cells satisfying the sorting criteria, only one cell at a time can be sorted for cloning purposes or direct (microchemical) single cell analysis.

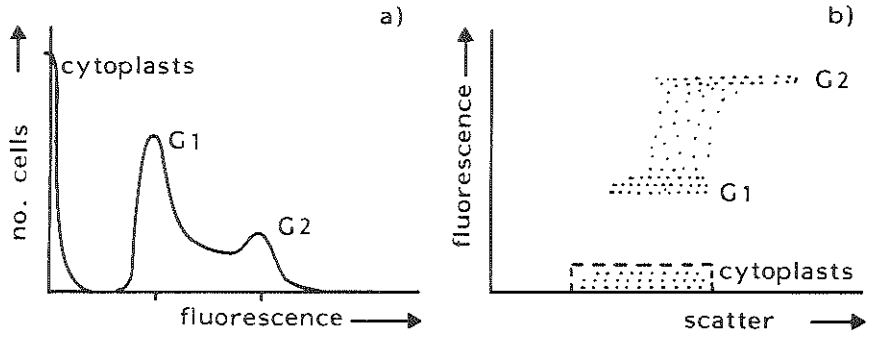

Figure 1.2 Example of a histogram and a dot plot. In this example a mixture of enucleated cells (cytoplasts) and cells with nuclei is analyzed. The DNA in the nuclei is stained with a fluorescent DNA stain. The optical signals generated for each cell (see figure 1.1) are converted into electrical pulses and are processed by the electronics of the cell sorter. In a histogram (a) all pulses are collected and displayed according to size. The number of cells with a certain amount of DNA can be obtained from this histogram. The nonfluorescent cytoplasts are distinguished from the fluorescent nucleated cells. The nuclei from the cells contain various amounts of DNA according to their position in the cell cycle (G1 and G2 are indicated). In a dot plot (b) each dot represents one analyzed cell. In this way two parameters of the same cell (in this case fluorescence and light scatter) can be plotted. In this display cytoplasts and nucleated cells form two distinct clouds of dots. For sorting purposes the limits of the fluorescence and light scatter pulses for cells to be sorted can be evaluated (indicated for cytoplasts).

1.3 Use of sorting capabilities.

Some measurements can be made on cells as such (forward and 90° light scatter and autofluorescence) but in most cases cells have to be labelled with fluorescent probes. Many different probes labelling various parts of the cells (for example, DNA, mitochondria, membrane antigens ,see review (13)) are available and can be combined with the different options of the FACS. In this way a large number of different possibilities exist for the isolation of cells by flow sorting. This is illustrated by the following examples.

1.3.1 Isolation of specific cells

Blood cells. There are a great number of reports on the enrichment of various cell types from blood and bone marrow. Forward and 90° light scatter, specific antibodies, stainability by the DNA stain Hoechst 33342 and autofluorescence have been used to

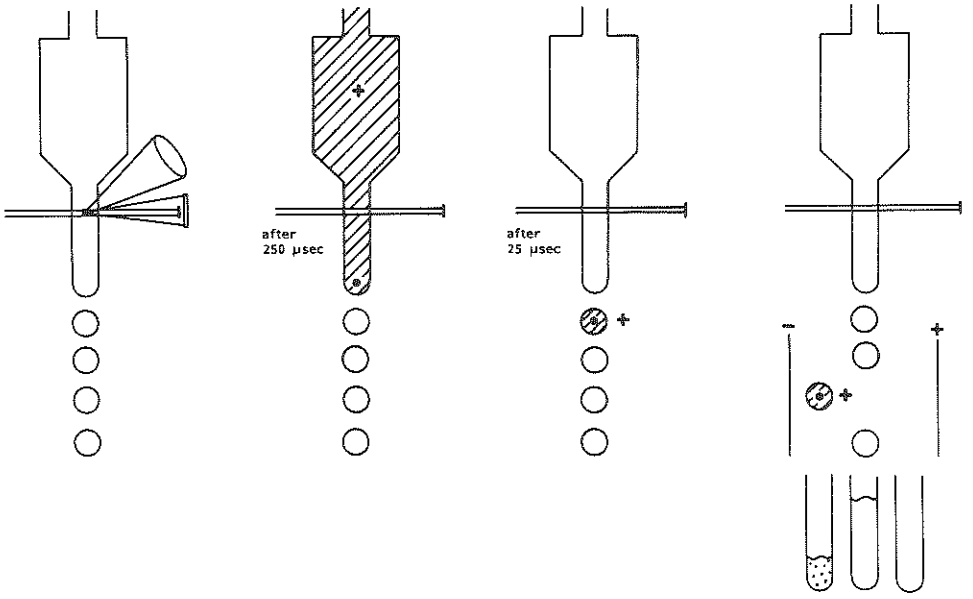

Figure 1.3 Principle of flow sorting. Ultrasonic vibration causes breakup of the fluid jet into uniform droplets. Approximately 250µsec. after passing the laser beam a cell reaches the point where droplet formation occurs. During this time it is decided whether this cell is to be sorted. If the cell is to be sorted, the fluid jet is electrically charged for a short period of time. In this way a charged droplet is formed. This droplet passes between two electrically charged plates and is deflected. Deflected cells are collected separate from the main stream.

discriminate and sort cells (see for examples 18,19,20,21, 22,23,24,25).

Tissue cells. The isolation of cells from tissues requires physical disruption and enzymatic digestion in order to obtain a single cell suspension suitable for sorting. Such treatments generally weaken the cells and complicate isolation by flow sorting. A number of different cell types have been isolated and enriched using various cellular characteristics (see table 1.1).

1.3.2 In vitro cultured cells.

Many applications concern the isolation of spontaneously altered cells or cells that have undergone some form of manipulation.

Table 1.1 Tissue cells isolated/enriched by flow sorting.

cell type	cellular property	fluorescent labelling	light scatter	ref.
mouse testis cells	DNA	Hoechst 33342		29
rabbit type II pneumocytes	lamellar bodies	phosphine-3R	+	33
gonadotrophs (anterior-	LH-secretion	LH-antibody coupled to microspheres	+	32
pituitary, rat)	hormone receptor	fluorescently labelled analog		27
epidermal Langerhans cells (guinea pig, human)	Ia-antigen	indirect immuno-fluorescence with anti-Ia		31
B-cells from islets of			+	30
Langerhans (rat)	response to glucose	FAD and NAD(P)H autofluorescence		26
chick embryonic neural retina (various cell	DNA	Hoechst 33342 + quenching by BrdU		28
types)	protein	RITC/Fluram		

Spontaneous alteration. Myeloma variants expressing a variant surface immunoglobulin and a lymphoma cell expressing variant H-2K could be isolated using fluorescent antibodies and several rounds of selection with the FACS ending with cloning (34,35). Cinese hamster ovary cells with spontaneously increased content of dihydrofolate reductase gene were isolated after 10 successive rounds of growth and sorting using fluoresceinated methotrexate as fluorescent stain (146).

Hybridomas. Using fluorescent microspheres coupled with antigens it was possible to select and directly clone hybridomas producing monoclonal antibodies against the antigen (36). Clones of T-cell hybridomas obtained from the fusion of activated T-cells and T-lymphoma cells were isolated after a double selection round using fluorescent antibodies to cell surface antigens and Hoechst fluorescence (37).

Cells transformed with DNA. L-cells transformed with human (lymphocyte) DNA stably expressing human membrane antigens, HLA and β2-microglobulin have been isolated in several rounds of selection using antibodies against these antigens (38). Similarly L-cell transformants expressing the human transferrin receptor have been selected (39).

Fused cells. When two different cell types are fused, isolation of heterokaryons is possible after cell fusion by labelling the parental cells with different colours using fluorescent microspheres (40,48), fluorescent membrane probes (41,53, this thesis) or by direct labelling with FITC and RITC (42,52). Isolation of heterokaryons is also possible measuring the resonance energy transfer occurring between two different membrane probes (43,44). When cells and cytoplasts are fused, selection and cloning of cybrids can be done using fluorescent beads and the DNA stain Hoechst 33342 with dual laser excitation (45, this thesis).Rhodamine 123, a fluorescent dye specifically staining mitochondria has also been used to label cytoplasts, whereafter cybrids were distinguished by this fluorescence and light scatter (46).

Macromolecules can be introduced in cells by fusion with erythrocyte ghosts. Cells having fused with specific numbers of ghosts filled with FITC-labelled albumin have been isolated by flow sorting (47).

Cocultivated cells. Separation of cells from mixed cell cultures after cocultivation was possible when the different cells were labelled with two colours fluorescent beads (41,166). Such a separation is also possible using an artificially created difference in DNA content by staining the cells with the vital DNA stain Hoechst 33342 (49, this thesis).

1.3.3 Subcellular fragments.

Cytoplasts. After cell enucleation, cytoplasts can be separated from isolated nuclei and intact cells using Hoechst 33342 (50,51, this thesis).

Chromosomes. A review describing and discussing the various possibilities and merits of chromosome analysis and sorting has recently appeared (16). Separations are based on size differences and differences in AT/CG content using various DNA stains. For two applications sorting can be useful; subchromosomal gene mapping and the establishment of chromosome specific gene librararies. Due to the small size of the chromosomes high intensity lasers generally are needed.

1.3.4 Sorting of cells for direct analysis.

Many cellular properties cannot be assayed directly using specific fluorescent probes. Using the sorter as a single cell depositor,

these sorted single cells can be analysed by microchemical analysis to reveal possible heterogeneity in the original cell population (55,56).

1.4 Summary.

Flow cytometers and sorters measure properties of single cells and make it possible to detect heterogeneity with respect to these properties within a cell sample. Based on such an analysis sorters further allow the separation of subpopulations. A large number of applications in cell biology and clinical medicine have resulted from the use of the technique.

The FACS II cell sorter employs the principle of laminary sheath flow for the precise location of cells in the middle of a fluid stream. A laser beam crosses the fluid stream, producing a sensing region through which the cells are passing one by one. In this way fluorescence and light scatter can be detected for each cell. Electrostatic droplet deflection is used for sorting. Possibilities also exist for two colour analysis, polarization measurement, two laser excitation and sorting of one cell at a time.

Light scatter and autofluorescence can be measured without staining but usually fluorescent labelling is required. Flow cytophotometric cell separations have exploited various cellular properties; DNA content, protein content, secretion of a product, lipid content, specific membrane antigens and mitochondria, using various specific fluorescent probes. Moreover a-specific labelling with microspheres and membrane probes is possible.

Chapter 2.

Cell differentiation studied by cell hybridization.

2.1 Introduction.

All higher plants and animals are constructed from a large variety of cell types. For example slightly over two hundred different cell types are listed in a catalog for man (57). All these different cells arise in a regular way from one single precursor cell, the fertilized egg. The proces by which stable differences arise between cells is called cell differentiation (157).

The different cells differ from each other because they synthesize and accumulate different sets of proteins which are coded by genes in the DNA of the cells. With a few exceptions all the different cell types contain the same genes. Moreover during the process of cell differentiation these genes are not irreversibly altered. This was already demonstrated by Gurdon (58) and recently confirmed by the elegant experiments of DiBerardino et al. (59) who succeded in obtaining a swimming tadpole from the nucleus of a fully differentiated frog erythrocyte by serial transplantation into enucleated frog eggs. Cell differentiation thus has to do with selective use of the information in the DNA.

Already at a very early stage in embryonic development essential regulation processes take place. In the mouse, first signs of differentiaton are seen at the 32 cell stage with the formation of the blastocyst with differentiated trophectoderm cells (60).

2.2 Analysis of cell differentiation by cell hybridization.

The method of somatic cell hybridization is used to study cell differentiation. Hybridization of two different cells permits the confrontation of the two genomes within a single cell and is used to study the mechanisms responsible for the induction and maintenance of the differentiated state.

When two different cells are fused a heterokaryon is formed. Proliferating hybrid cells, containing chromosomes from both parental cells within a single nucleus, can be isolated as growing colonies after some weeks by employing selective media (66). Apart from combinations of two cells, a cell can also be fused with an enucleated cell (cytoplast) forming a cybrid. Also the nucleus of a cell (karyoplast) can be fused with a whole cell forming a karyohybrid or with a cytoplast producing a reconstituted cell.

Since the introduction of the hybridization technique a large amount of different cells have been fused with each other (for reviews see, 63,64,65,66,67) and the following observations concerning the expression of differentiated functions have been made

(see also 67).
1. Coexpression. Coexpression of both parental forms of homologous tissue specific functions is observed when the two parents expressed them prior to fusion.
2. Extinction. Extinction of the expression of tissue specific functions is generally observed when only one of the parents expressed them prior to fusion.
3. Reexpression. After some time of cultivation reexpression of previously extinguished functions can occur.
4. Gene dosage effect. Doubling the ploidy of the expressing parent may lead to the formation of hybrids in which extinction is not observed.
5. Activation. When extinction does not occur or is only partial, activation of the previously silent gene from the non-expressing parent may lead to the production of the homologous tissue-specific product.

As pointed out by M.C.Weiss (67) all observations concerning the expression of tissue specific proteins in fused cells are compatible with the notion that both parental genomes continue, after fusion, to synthesize the same regulatory factors that they produced before fusion and that the final effect is a reflection of the balance of these factors.

2.3 Heterokaryons and cybrids.

Both heterokaryons and cybrid cells have been used to investigate, more directly than is possible with hybrids, the factors causing activaton or extinction. With heterokaryons effects are usually studied over a period of one to several days after fusion, while with cybrids there are two possibilities. Either short term effects as with heterokaryons can be studied, or long term effects (proliferating cybrids) which requires that the induced change is self perpetuating. Activation as well as extinction has been observed in both fusion systems.

Heterokaryons. In heterokaryons the nuclei of both parental cells are within the same cytoplasm and can interact over the entire experimental period. Published results with heterokaryons showing the phenomena of activation and extinction of tissue specific functions are listed in table 2.1.

Activation was found when (human) amnion cells were fused with (mouse) myotubes.In that case human myosin chains and creatine kinase activity appeared (122). In these fusion products the myoblast nuclei were in vast excess. Also activation of rat myosin light chains was observed when (rat) myoblasts were fused with closely related (chicken) myocytes (121). Thus activation has thusfar only been observed when gene dosage was very much in favor of the activating parent or with intralineage heterokaryons.

Extinction has been observed more often than activation.

Table 2.1 Activation and extinction in heterokaryons.

marker	parental cells fused	ref.
Activation		
myosin light chains	myocytes (chick) myoblast (rat)	121
myosin light chains 1,2 creatine kinase	myotubes (mouse) amnion cells (human)	122
creatine kinase surface antigen 5.1H11	myotubes (mouse) fibroblasts (human)	167
Extinction		
phagocytosis, lysosomal enzymes, membrane ATPase	macrophage (human) melanoma (human)	110–113
hemoglobin	A9 (mouse) erythroblast (chick)	114
albumin	fibroblast (mouse) hepatoma (rat)	116
fibronectin matrix formation	fibroblast (human) epithelial cells (MDCK)(canine) (Hela)(human)	117
dopa oxidase	fibroblast (chick) melanoma (mouse)	118
myosin light chain,α-bungarotoxin binding sites, CPK, myotube formation	fibroblast (rat) myocytes (chick)	119
κ-light chain production	hepatoma (rat) lymphoma (human)	52
immunoglobulin	non lymphoid cells myeloma (human)	120
TAT inducibility	hepatoma (rat) epithelial cells (mouse)	115

In many cases fibroblasts have been used as one of the parental cells and these cells can repress various differentiated functions. It should be noted that cross extinction did not occur in hepatoma x lymphoma heterokaryons where (lymphoma specific) κ-light chain production was extinguished while (liver specific) tyrosine aminotransferase (TAT) expression continued. Some evidence for a gene dosage effect was also obtained in fibroblast x hepatoma heterokaryons where extinction was not observed when the hepatoma nuclei were present in excess (116).

Cybrids. With cybrids both permanent and short term effects have been observed. DMSO induced hemoglobin synthesis in Friend cells was permanently extinguished after fusion with neuroblastoma- or fibroblast cytoplasts (106). Permanent activation of the liver specific enzyme phenylalanine hydroxylase was found after fusion of Friend cells with hepatoma cytoplasts (107). It should be noted however that cells expressing the enzyme were selected for. Other liver specific functons were not induced (158). Recently evidence was obtained that RNA is the factor responsible for induction (159). Temporary activation of the liver specific enzyme TAT was found in reconstituted cells formed from fibroblast karyoplasts and hepatoma cytoplasts (108) and temporary extinction of albumin secretion was observed in hepatoma cells after fusion with fibroblast cytoplasts (109).

2.4 Teratocarcinoma cells.

Teratocarcinomas are malignant tumors of ovarian or testicular origin, characterized by numerous differentiated tissues which may represent all of the three primary germ layers. Stem cells can be isolated from teratocarcinomas and cultured *in vitro* and are called embryonal carcinoma or teratocarcinoma (stem) cells. Established teratocarcinoma cell lines retain the capacity to differentiate both *in vivo* and *in vitro* although the differentiation potential varies with different cell lines. Teratocarcinoma cells bear a close resemblance to normal early embryonic cells but there is some uncertainty about the exact embryonic equivalent. Similarities exist with cells of the embryonic inner cell mass and with pluripotent cells in the embryonic ectoderm (for review see 61). Recently is has become possible to establish cultures of pluripotent cells directly from embryonic cells (62,160,161). These pluripotent cells resemble teratocarcinoma cells.

Teratocarcinoma cells are used to study various aspects of early embryonic development. Their resemblance to embryonic cells makes them interesting cells for cell hybridization studies.

2.5 Teratocarcinoma hybrids.

Hybrids between (mouse) teratocarcinoma cells and a large number

of different differentiated cells have been produced and studied: fibroblasts (68,69,70,71,72,73,74,75,76,99,100,103), primary lymphoid cells (77,78,79,72,80,81,82,83,101,102), lymphoid tumor cells (79,82,84,81), Friend cells (85,86,68,87,88,90), neuroblastoma cells (89,75), hepatoma cells (91,92,93,94,95,103), melanoma cells (96,97), endoderm cells (98),villus cells (164) and lens epithelial cells (102). Teratocarcinoma x teratocarcinoma hybrids have also been isolated (162,163,102).

These experiments show that teratocarcinoma x differentiated cell hybrids may have either the teratocarcinoma- or the differentiated phenotype. Occasionally phenotypes other than parental have been found (76,81,82,92,95)

The final phenotype may to some extent be influenced by the type of differentiated parent. Thus fibroblast hybrids in nearly all cases resembled fibroblasts, while primary lymphoid-cell hybrids nearly always resembled teratocarcinoma (see also 72). However in crosses with other differentiated cell types hybrids resembling the teratocarcinoma as well as the differentiated parent have been observed, sometimes within one experiment (86,88,97).

The phenomena of extinction, reexpression, gene dosage and activation (see above) have been found with the teratocarcinoma hybrids as well.

ad.2 Extinction. Extinction of differentiated properties can occur (hybrids resembling teratocarcinoma). In addition in hybrids resembling the differentiated parent extinction of undifferentiated properties of the teratocarcinoma parent has been demonstrated.

ad.3 Reexpression. After in situ differentiation (injection of hybrid cells into blastocysts and chimera development) of teratocarcinoma x (rat) hepatoma hybrids, (developmentally regulated) rat gene products glycerol-3 phosphate dehydrogenase and albumin reappeared (94). Some thymoma x teratocarcinoma hybrids resembling fibroblasts later gave rise to cells with teratocarcinoma morphology (82). This can be considered as reactivaton of the undifferentiated phenotype.

ad.4 Gene dosage. While hybrids from diploid teratocarcinoma cells and Friend cells resembled Friend cells, tetraploid teratocarcinoma cell x Friend cell hybrids resembled teratocarcinoma (86).

ad.5 Activation. Differentiated properties coded for by teratocarcinoma cell genes have been found in various hybrids (H2 antigen in fibroblast hybrids (72,70) and hemoglobin in Friend cell hybrids (90)). Also activation of a gene contributed by the differentiated parent coding for an "undifferentiated" gene product has been observed (t^{12} antigen, expressed only during embryogenesis) in teratocarcinoma x thymocyte hybrids (80).

Teratocarcinoma x teratocarcinoma hybrids resembled teraotocarcinoma. Fusion of "nullipotent" with "pluripotent" cells may lead to pluripotent hybrids (163). It is of interest to note that also fusion of nullipotent teratocarcinoma cells with differentiated cells may lead to pluripotent hybrids (101,102).

Apart from extending the general observations made with other types of hybrids the use of teratocarcinoma cells as one of the

parental cells in cell hybrids has not yielded essentially different information.

There are a number of problems associated with the analysis of cell differentiation with proliferating hybrids in general and more specifically with teratocarcinoma hybrids.

1. Chromosome losses occur which may lead to secondary variations in phenotype.
2. Hybrids with a differentiated phenotype might not be detected since proliferating hybrids are selected and differentiation often is accompanied by a cessation of proliferation.
3. On the other hand hybrids with a differentiated phenotype might occur because of the chemical selection process, since during this process the environment of the cells might be such that *in vitro* differentiation of originally undifferentiated hybrids is promoted. Such an event might also give rise to hybrid phenotypes other than parental.
4. Teratocarcinoma hybrids are generally isolated at very low frequencies. Although heterokaryons are formed, combinations with some differentiated cells do not give rise to proliferating hybrids at all. Both developmental and intraspecies differences between the two cell parents may contribute to this phenomenon (104,105).

The use of undifferentiated teratocarcinoma cells in cell hybridization studies has not led to hybrids with a predictable phenotype. With a view to the problems mentioned above it is possible that with the heterokaryon system, where these problems do not play a role, predictable results can be obtained. Gene expression in teratocarcinoma heterokaryons has been subject of this thesis and of other studies in this laboratory (Chapter 3).

2.6 Summary.

Many of the cells of a higher organism are strikingly distinct both in morphology and function. Since all these different cells have the information for the same proteins in the same DNA, this information must be used in different ways in different cells. Cell differentiation is the process by which stable differences between cells arise.

Cell hybridization can be used to study cell differentiation. In fused cells the following observatons concerning the expression of differentiated functions have been made; coexpression, extinction, reexpression, gene dosage effect and activation. All observations concerning the expression of tissue specific functions in hybrid cells are compatible with the notion that both parental genomes continue, after fusion, to synthesize the same regulatory factors that they produced before fusion and that the final effect is a reflection of the balance of these factors. The presence of such factors has been demonstrated most directly in heterokaryons and cybrids.

Teratocarcinoma cells resemble undifferentiated early embryonic cells and are therefore interesting fusion partners. Hybrids between teratocarcinoma cells and differentiated cells resemble teratocarcinoma cells or differentiated cells or in some cases neither parent. The phenotype of these hybrids cannot be predicted. It is possible that this is caused by difficulties inherent to the analysis of proliferating hybrids. The use of heterokaryons might prevent these problems and give more meaningful results.

Chapter 3.

Introduction to the experimental work and discussion.

3.1 Introduction.

As was discussed in chapter 1, a cell sorter is a versatile instrument that can be applied in many different ways in cell biological studies. Likewise a number of aspects of the in vitro differentiation of terarocarcinoma cells can favourably be studied using the capabilities of the cell sorter. The experiments described in this thesis were restricted to such aspects.

The experimental work can be subdivided in four parts.

Fluorescence polarization measurements. Undifferentiated teratocarcinoma cells were chemically induced to differentiate, and the mobility of membrane embedded fluorescent probes was studied flowcytophotometrically in undifferentiated and differentiated cells.

Cocultivation. Undifferentiated teratocarcinoma cells were cocultivated with differentiated cells to investigate whether this would result in induction of differentiation of the teratocarcinoma cells.

Cybridization. Undifferentiated teratocarcinoma cells were fused with enucleated differentiated cells to see whether this would lead to a stable epigenetic induction of differentiation.

Heterokaryon analysis. Undifferentiated teratocarcinoma cells were fused with differentiated cells to study possible activation of silent teratocarcinoma genes in the fused cells.

The last three groups of experiments all investigate the existence of factors in differentiated cells, capable of activating teratocarcinoma cell differentiation. They also have in common the use of the sorting capabilities of the cell sorter to select cells from mixed populations for further experimentation.

3.2 Fluorescence polarization (appendix paper 6).

With fluorescence polarization (FP) measurements the degree of mobility of membrane embedded fluorescent probes can be determined (for a detailed explanation of the technique see 142). This mobility is a reflection of the "fluidity" of the membranes in which the probes are located (143). A proposed functional role for the plasma membrane fluidity is that it regulates the exposure of membrane proteins at the outside of cells (e.g. antigens, hormone receptors)(144). In such a way changes in plasmamembrane fluidity might play a role in the process of cell differentiation. In the teratocarcinoma cell model system (F9 cells) evidence for a decrease in plasmamembrane fluidity upon chemically induced differentiation had been obtained by Searls and Edidin (145), with the technique of fluorescence photobleaching recovery. In the experiments in appendix

paper 6 the same cell system was investigated with flowcytophotometric FP measurements.

First the performance of the cell sorter with respect to this type of measurement was tested and a procedure was developed in order to be able to obtain quantitative results. It was found that correct mean FP values could be obtained.

FP measurements on teratocarcinoma cells were done with six different membrane probes: diphenyl hexatriene (DPH), tetramethyldiphenyl hexatriene (TMA-DPH) and a set of 4 anthroyloxystearate probes. Different probes can yield different information since their microenvironment will vary. With the 4 anthroyloxystearate probes no changes in FP were found upon differentiation, while an increase was seen both with DPH and TMA-DPH. Little or no heterogeneity was observed in the FP distributions of the differentiated cells; all cells shifted towards higher FP values. Reproducible effects were only obtained with a specific induction protocol (10^{-7} M retinoic acid + 10^{-3} M dibutyryl-cAMP for four days followed by another two days in the absence of inducers). Although under these conditions considerable heterogeneity exists in the cultures this is clearly not reflected in the FP profiles. Other induction protocols sometimes led to morphological differentiation without an increase in FP. Together these results suggest that the increase in FP upon differentiation is a secondary effect. Recently Jetten et al. (147) performed static (DPH) FP measurements on differentiating OC15-S teratocarcinoma cells and found a very fast increase in FP (within 3 days), dependent on differentiation. Their suggestion, on the basis of their results, of an important role for the decreasing "membrane fluidity" in the differentiation proces, is not supported by our experiments.

The increase in FP as seen with DPH and TMA-DPH is in line with the observation of a decrease in plasmamembrane fluidity upon F9 differentiation by Searls and Edidin (145). However it is known that DPH does not remain in the plasmamembrane but also penetrates cells (see for example 148,149,150). Our microscopical observations also showed that DPH penetrated the cells. This makes it impossible to draw a conclusion about the cellular localization of the observed FP effect. TMA-DPH is a charged analog of DPH, designed to make diffusion to the inside of the cells more difficult. In our experiments clear plasmamembrane labelling was observed with this probe, but also some intracellular staining although less complex than with DPH. Recently Khury et al. (151) reported that TMA-DPH remains specifically localized on the cell surface for approximately 25 min., whereafter penetration starts. Since TMA-DPH has less complex staining characteristics than DPH, while the FP shift was found similar to DPH it clearly is a more useful probe for FP measurements on living cells.

The experiments described in this section thus gave the following results.

1. A quantitative procedure for FP measurements on the FACS was developed.
2. An increase in FP was found upon F9 teratocarcinoma

differentiation with the probes DPH and TMA-DPH.
3. This increase is a secondary effect which is observed after differentiation has occurred.
4. The usefullnes of TMA-DPH as FP-probe, instead of DPH, was demonstrated.

3.3 Cocultivation (appendix paper 5).

Classically, cell interactions have been recognized as being important in regulating developmental phenomena such as primary embryonic induction and organ formation (123,124). In early development, cell interactions are involved in the allocation of cells destined for different fates and in controlling the expression of cell-specific gene products (125,126). In the teratocarcinoma cell model system several lines of evidence show that the *in vitro* and *in vivo* environment of the teratocarcinoma cells can influence their behaviour. Aggregation of teratocarcinoma cells in various ways (growth in densely packed colonies, dense monlayer culture and as suspended aggregates) may cause spontaneous differentiation *in vitro* (73, 128,129). Also cell interactions can modulate chemically induced teratocarcinoma cell differentiation into parietal or visceral endoderm (132). Furthermore in vivo blastocyst injection of teratocarcinoma cells may cause these cells to differentiate in a regular way, with differentiated cells from teratocarcinoma cell origin contributing to the embryo (130,131).

Cocultivation is a simple way to study cell-cell interaction *in vitro*. We were interested to cocultivate PCC4AZA1 teratocarcinoma cells with endoderm cells for the following reasons. Firstly endoderm cells are differentiated cells that can readily develop upon teratocarcinoma cell differentiation and secondly, metabolic cooperation (133) was observed by us between these two cell types, indicating the presence of functional gap-junctions. Thus in dense cocultivation mixtures the cytoplasms of all cells are interconnected and diffusion of small molecules is possible (channel diameter 1.6-2.0 nm, (134)). In addition in principle interaction of the cells is also possible through factors which are secreted by the cells and diffuse through the medium. A cell sorter can be of use in these experiments for the separation of the two cell types after cocultivation provided that there is a difference between them, that can be detected flowcytophotometrically.

In the experiments described in appendix paper 5, the endoderm cells were made tetraploid to create a permanent difference in DNA content between these cells and the (near diploid) teratocarcinoma cells. With the vital fluorescent DNA stain Hoechst 33342 this difference could be detected by the cell sorter and could be used for sorting of viable teratocarcinoma cells (see figure 3.1).

In the actual experiments teratocarcinoma cells were sorted out, after being cocultivated for 24 days, and the proteins synthesized by the sorted cells were analyzed with the technique of two dimensional gelelectrophoresis (135). The 2-D pattern obtained for the sorted

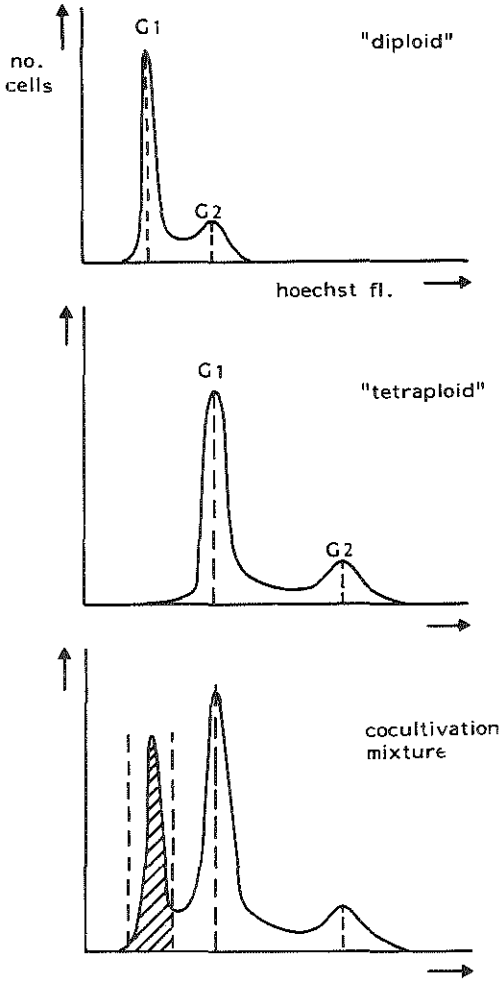

Figure 3.1 Sorting of cocultivated "diploid" and "tetraploid" cells. The cells are stained with the fluorescent DNA stain Hoechst 33342. "Tetraploid" cells in G1 have the same amount of DNA as "diploid" cells in G2. "Diploid" cells in G1 can be sorted out from a cocultivation mixture.

cocultivated cells was identical with that of not cocultivated cells. Thus interaction of the PCC4AZA1 teratocarcinoma cells with differentiated endoderm cells does not appear to stimulate differentiation.

Consistent with this are the results of Rosenstraus and Spodaro who showed that in mixed aggregates of nullipotent and pluripotent teratocarcinoma cells the nullipotent teratocarcinoma cells were not induced to differentiate as were the pluripotent cells (136). Recently a number of papers have been published showing that in some cases the interaction of teratocarcinoma cells with endodermal cells may result in inhibition of differentiation and stimulation of

growth. Thus aggregation of teratocarcinoma cells with endodermal cells resulted in mixed aggregates with an inner core of teratocarcinoma cells and an outer layer of endodermal cells in which teratocarcinoma cell differentiation to endoderm was inhibited. Likewise F9 teratocarcinoma cells in such aggregates failed to differentiate in response to retinoic acid (137). Also in cocultivation experiments feeder cells of endodermal origin (retinoic acid induced PC13 END cells) stimulated clonal growth of PC13 teratocarcinoma cells (138).

The experiments described in this section thus had the following results.

1. A method was developed for the reisolation of cells from a mixed culture.
2. Cocultivation of teratocarcinoma cells with endoderm cells did not induce differentiation of the teratocarcinoma cells.

3.4.Cybridization (appendix papers 1,2 and 4).

As was discussed in chapter 2, hybridization studies in general indicate the existence of cellular regulatory factors and the phenomena of activation and extinction have also been observed in cybridization experiments.

The experiments in appendix papers 1,2 and 4 are about the production, isolation and analysis of proliferating cybrid cells. Teratocarcinoma cells were fused with neuroblastoma- and endoderm cytoplasts to see whether this would induce differentiation.

Using the cell sorter, procedures were developed for cytoplast isolation and for selection and cloning of cybrids. Appendix papers 1 and 2 decribe the isolation of cytoplasts (see figure 3.2) A sorting step was added to the existing technique of density-gradient enucleation (139) and in this way an essentially pure cytoplast population (>99%) could be obtained (appendix paper 1). A simplified procedure using Percoll as gradient material was then developed (appendix paper 2). It was also found that the Hoechst dye used for staining could be washed away, allowing the cytoplasts to be fused with cells without impairing growth of the resulting cybrids. In addition it was shown that cytoplasts containing green fluorescent beads could be isolated this way, creating possibilities for the isolation of cybrids by flow sorting. In appendix paper 4 a method for the isolation of proliferating cybrid cells by dual laser flow sorting is described (see figure 3.3) Teratocarcinoma cells were fused with neuroblastoma- and endoderm cytoplasts. Cloned proliferating cybrids (16 neuroblastoma cybrids and 8 endoderm cybrids) were isolated and their 2D-protein pattern was determined. Comparison with the 2D-patterns of the parental cells showed that all cybrids resembled the teratocarcinoma parent.

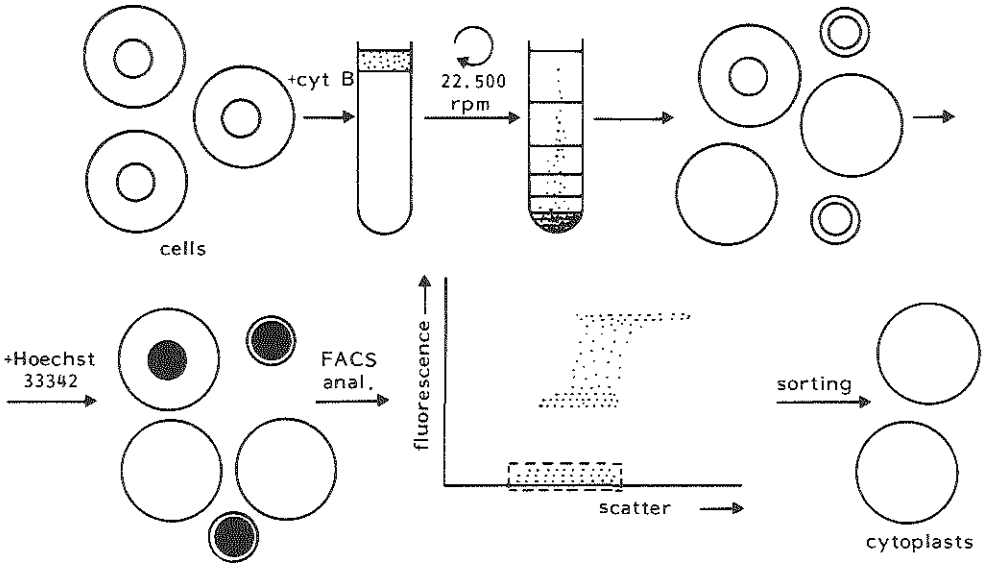

Figure 3.2 Isolation of cytoplasts by flow sorting. Cells are incubated with cytochalasin B and are then centrifuged through a density gradient. The resulting mixture of enucleated cells (cytoplasts), intact cells and isolated nuclei is stained with the fluorescent DNA stain Hoechst 33342. Cytoplasts are non-fluorescent and can be sorted out.

The methods for cytoplast isolation and cybrid formation have been used extensively in complementation studies concerning DNA repair and lysosomal storage diseases (154,155). Recently a procedure for cybrid isolation by sorting has been published, using the fluorescent dye rhodamine 123 to label the mitochondria of the cytoplasts (46). It should be noted that the retention time of rhodamine 123 in the mitochondria varies with different cell types (156) and that this method is only useful for cells that do not rapidly lose the dye.

The fact that teratocarcinoma cells, although capable of differentiating in the direction of the cytoplasmic donor cells, were not induced to differentiate upon cybridisation will be discussed in conjunction with the results obtained with the heterokaryons (see 3.6) This result is however in line with other observations with teratocarcinoma cybrids. Proliferating cybrids of teratocarcinoma cells and melanoma (96), Friend cell (74), fibroblast (74) and myoblast (140,141) cytoplasts all resembled teratocarcinoma cells. Short term experiments with melanoma cytoplasts showed that expression of embryonic antigens was transiently suppressed (97).

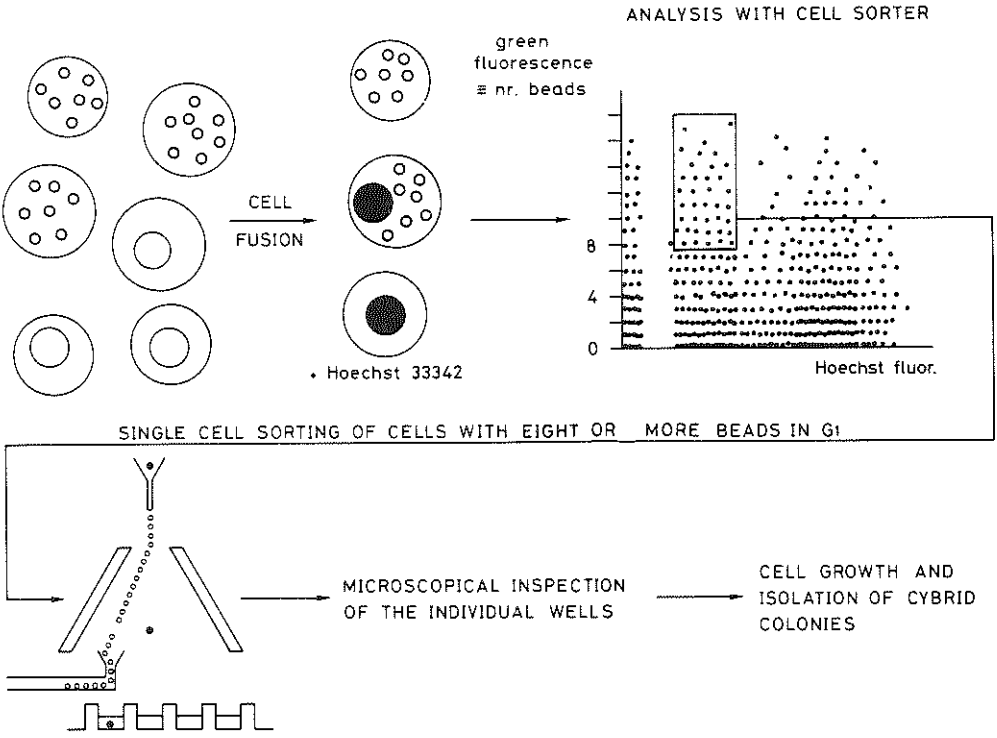

Figure 3.3 Isolation of proliferating cybrid cells by flow sorting. Cytoplasts labelled with green fluorescent beads are fused with cells. The fusion mixture is stained with the fluorescent DNA stain Hoechst 33342. Cybrids are cells with both a fluorescent nucleus and fluorescent beads. Using two laser excitation (not indicated), cybrids can be distinguished from unfused parental cells and cytoplasts. Cybrids in G1 and containing eight or more beads are sorted one by one into separate wells of a culture dish. After microscopical inspection of the separate wells, cells are grown up and cybrid colonies are isolated for further subculture and analysis.

With the experiments described in this section the following results thus have been obtained.

1. A method for the purification of cytoplasts by flow sorting was developed.
2. A method for the isolation of proliferating cybrid cells was developed.
3. Fusion of (PCC4) teratocarcinoma cells with the cytoplasm of differentiated cells does not lead to a stable induction of differentiation, that can be observed in proliferating cybrids.

3.5. Heterokaryon analysis (appendix papers 2 and 7).

As was discussed in chapter 2, heterokaryon analysis is the most direct way to study activation and extinction of differentiated properties. However analysis of these phenomena in heterokaryons is complicated by the presence of a background of unfused cells. When the differentiated property can be analyzed in the heterokaryons at the single cell level (for example with specific antibodies), no purification of heterokaryons is needed. If single cell analysis is not possible unfused cells will have to be eliminated. The required degree of purity depends however on the phenomenon studied. To be able to detect extinction, the differentiated property has to be quantified and the degree of purity therefore should approach 100% (no contamination of the heterokaryons with unfused parental cells). To be able to detect activation the first requirement is that the homologous differentiated gene products can be identified, but also in this case obviously purity should be as high as possible. The experiments in appendix papers 2 and 7 are about the purification of heterokaryons and the analysis of mouse teratocarcinoma x human fibroblast heterokaryons.

A method for the purification of heterokaryons by two-colour flow sorting has been developed in this laboratory using red- and green fluorescent microspheres (40)(see figure 3.4). Since the PCC4 teratocarcinoma cells used in this study do not sufficiently take up such beads, an alternative way to label the cells was developed (appendix paper 2). Red- and green fluorescent membrane labels were synthesized and tested on fibroblasts. With a procedure similar to the one used with bead labelled cells, fibroblast heterokaryons could be isolated with a purity greater than 90%. The advantage of these membrane labels is that they can be applied to all cell types and furthermore that the intensity of staining can readily be adjusted in order to obtain the optimal ratio of the intensities of the two colours fluorescence (see appendix paper 2). With these labels mouse teratocarcinoma x human fibroblast heterokaryons could be isolated with purities of around 80% (appendix paper 7).

Until now there are two techniques for purification of heterokaryons; chemical selection with irreversible inhibitors (127) and flow sorting. No direct comparison of the two methods has been made. Chemical selection has some inherent difficulties (see also 54) the most important being the determination of the exact lethal dose of inhibitor and the fact that many dead and dying cells have to be eliminated before analysis can be performed. The method has succesfully been applied in the analysis of myoblast x myocyte heterokaryons (121). The advantage of the cell sorting procedure is that it can be applied for different cell types with only minimal adaptations, especially when fluorescent membrane labels are used. A similar procedure has been published recently. With direct labelling of the cells with FITC and RITC heterokaryons could be obtained with purities between 85 and 97% (42). A cell sorting procedure which uses resonance energy transfer between two membrane probes (which

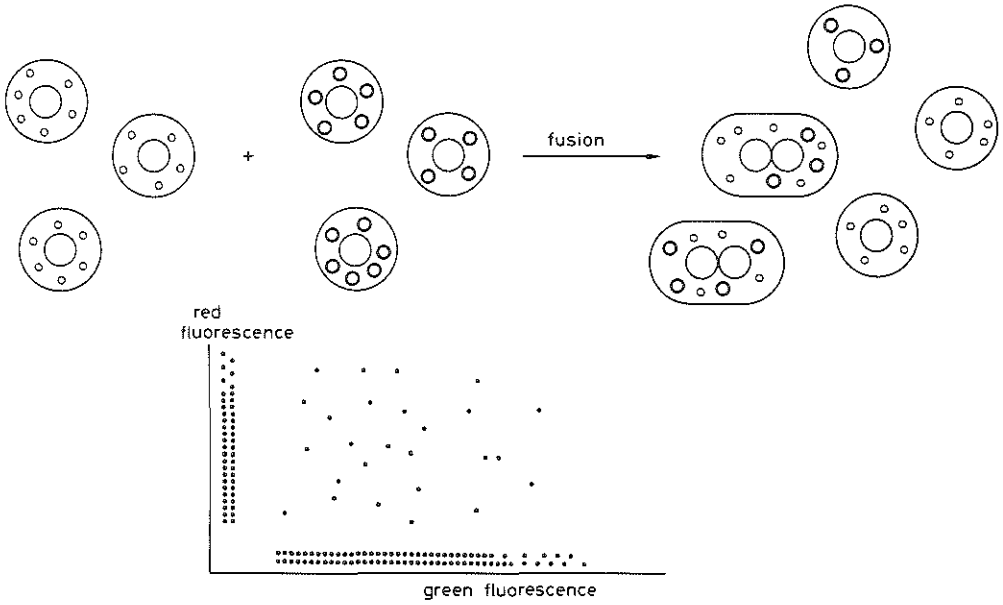

Figure 3.4 Heterokaryon isolation by flow sorting. Cells labelled with red- or green fluorescent microspheres are fused. Red-green heterofluorescent heterokaryons can be sorted out for culture and analysis. In a similar way heterokaryons can be isolated when the parental cells are labelled with red- or green fluorescent membrane labels.

occurs only when the probes are in close proximity in the membranes of the fused cells) has also been developed (44). The exact improvement with respect to two colour analysis in terms of purity and recovery of cells remains however to be determined.

In general three aspects –quantity,purity and viability– are important in these kinds of experiments. With respect to these points the procedure using membrane labels and flow sorting as described in appendix paper 7 can be summarized as follows.

1.Quantity. The amount of heterokaryons that can be isolated depends in the first place on the fusion percentage and is limited by sorting time. To give an indication: with a practical sorting time of 1 hour, a maximal working speed of 3000 cells/s, 10% fusion and 50% sorting recovery, 500,000 heterokaryons (ca. 0.3 μg cell protein) can be isolated starting with 10^7 cells before sorting. This is an amount sufficient for many of our analyses.

2.Purity. The degree of purity depends primarily on the fusion percentage but generally is greater then 80%. Unfused cells present

in small clumps are unavoidable in low percentage and are also registred as heterokayons (for example 1% cell clumps and 4% fused cells would lead to a maximal purity of 80%). As was discussed above the purity needed is dependent on the question asked.
3.Viability. Final cell viability is determined by the combined effects of staining, fusion and sorting. These effects have not been investigated systematically. Our experience with a number of different cell types is that fibroblasts can be considered as relatively "strong" cells and teratocarcinoma cells as relatively "weak" cells. For very weak cells like primary liver cells the method probably will not work. Fortunately heterokaryons may resemble the "stronger" parent, as was the case for our teratocarcinoma x fibroblast heterokaryons.

Fibroblasts are differentiated cells which sythesize and secrete large amounts of collagen type α1(I) and α2(I). These proteins are not synthesized by undifferentiated teratocarcinoma cells. The synthesis of these collagens was analyzed in the sorted fused cells by measuring radioactive collagen secreted into the culture medium and with immunofluorescence. Collagen synthesis continued in the heterokaryons and also in synkaryons (cells with fused nuclei, formed from heterokaryons). Mouse α2(I) type collagen could be distinguished from human α2(I), but in the fused cells no evidence could be obtained for expression of the mouse type collagen.

The two dimensional protein pattern of the sorted cells was analyzed and it was found that no proteins other than those synthesized already by the teratocarcinoma cells and the fibroblasts were expressed. However three proteins were synthesized in larger amount than in either of the parental cells and for one of these proteins it is very likely that this reflects the enhanced synthesis of a mouse protein. All three proteins are synthesized in large amounts by differentiated mouse endoderm cells and fibroblasts.

We have therefore not been able to detect the activation of a completely silent mouse gene in the fused cells. The results do show however that the teratocarcinoma cells do not suppres collagen synthesis, the differentiated characteristic of fibroblasts. In addition the 2-D pattern of the fused cells is altered. Thus all together the fused cells resemble the differentiated fibroblast parent rather than the undifferentiated teratocarcinoma parent.

With the experiments described in this section the following results thus have been obtained.
1. A method for purification of heterokaryons, applicable to teratocarcinoma cells, was developed.
2. Teratocarcinoma x fibroblast heterokaryons phenotypically resembled the differentiated fibroblast parent but no activation of silent teratocarcinoma genes was found.

3.6.Cellular factors regulating gene expression.Discussion.

The cocultivation, cybridization and hetero/synkaryon experiments described in this thesis were designed to investigate the possible existence of cellular (cytoplasmic) factors capable of inducing teratocarcinoma cell differentiation and the expression of differentiated properties not expressed by the teratocarcinoma cells.

From nuclear transplantation experiments it is clear that, with suitable manipulation, even the nuclei from fully differentiated cells can be reprogrammed to the state of a fully undifferentiated cell (59, for review see 152). However as was discussed in chapter 2 cellular substances regulating the expression of differentiated functions can also be studied in the cell fusion system.

If we look at these fusion experiments as a whole it is clear that the phenomenon of extinction is observed very often, while activation is only found when one of two conditions is fulfilled; a) an excess of expressing parental cell DNA is present (gene dosage effect) or b) the parental cells are related (intralineage fusion). For activation of a silent gene to occur it is necessary that, after fusion, the parent already expressing the product continues (at least partially) to express this product. These conditions can be taken into consideration in the design of cell fusion experiments to study activation.

Since teratocarcinoma cells are undifferentiated cells with a certain tendency to differentiate it should be possible, with these cells as a fusion partner, to study early events of cell differentiation. Given the normal direction of the differentiation process it is of interest to look for factors that are capable of extinction of undifferentiated teratocarcinoma gene products and of activation of teratocarcinoma genes that are not yet expressed (capable of induction of differentiation). In spite of the tendency to differentiate, cybridization studies with proliferating cybrids have failed to demonstrate induction of differentiation by cytoplasmic factors of differentiated cells. Also the phenotype of teratocarcinoma hybrids is largely unpredictable, but studies with hybrids have shown that activation can occur.

Both the cybridisation and cocultivation experiments described in this thesis have inherent limitations and can only demonstrate the presence of activating factors under certain conditions. Our finding with these cell systems of no induction of cell differentiation therefore does not necessarily mean that such factors do not exist. The heterokaryon system can give the most direct information, both about activation and extinction. However the investigation of heterokaryons experimentally is more difficult and besides this study and others in this laboratory (97,153) there are no reports about gene expression in teratocarcinoma x differentiated cell heterokaryons. Results obtained thusfar show that teratocarcinoma heterokaryons and synkaryons phenotypically resemble the differentiated parental cell. Embryonic antigens are suppressed (92) but expression of differentiated gene products from the

differentiated parental cells continues (collagen (appendix paper 7), MHC antigens (92) and albumin (153)). In addition activation of teratocarcinoma coded albumin has been observed in some teratocarcinoma x hepatocyte hetero- and synkaryons(153).

Unlike most heterokaryons with two differentiated cells the expression of differentiated gene products continues and therefore in these teratocarcinoma heterokaryons the prerequisite for activation, the continued expression of differentiated gene products, is fulfilled. Still even in heterokaryons generally no activation of silent teratocarcinoma genes is found despite the differentiation tendency of the teratocarcinoma cells. Increasing gene dosage or fusion with cells more closely related with regard to differentiation (for instance endoderm) can possibly lead to heterokaryons in which activation is observed. Such a heterokaryon system would be one of the very few ways to study early cell differentiation.

General summary.

Mammals like man and mouse contain a large number of different cell types. All these different cells are derived from one precursor cell, the fertilized egg. The process by which stable differences between cells arise is called cell differentiation. Teratocarcinoma cells have many properties in common with undifferentiated cells of the early embryo (blastocyst stage). Since these cells can be cultured *in vitro*, while retaining the capacity to differentiate both *in vivo* and *in vitro*, they constitute a usefull model system for the study of early cell differentiation.

A special feature of the experiments described in this thesis is the use of a fluoresence activated cell sorter. In this instrument cells in aquaeous suspension, one by one, pass a laser beam and at that moment produce optical (mostly fluorescence) signals. These signals are analyzed and can be used for selection of cells with specific fluorescent properties. Cells may be fluorescently labelled in various ways. In addition the cell sorter also has different options (for example two laser excitation and single cell sorting). The combination of the various ways of labelling and the specific configurations of the cell sorter allows a large number of different experimental designs.

Some aspects of the *in vitro* differentiation of teratocarcinoma cells may be advantageously studied by applying flow analysis and sorting and this is what this thesis is about.

The experimental work can be divided in four parts; fluorescence polarization measurements, cocultivation-, cybridization- and hybridization experiments.

Fluorescence polarization (appendix paper 6). Mouse teratocarcinoma cells can be induced to differentiate *in vitro* by treatment with chemical inducers. One of the parameters changing during cell differentiation is the "fluidity" of the plasma membrane. This "fluidity" can be determined indirectly,by measuring fluorescence polarization of membrane embedded fluorescent probes.

Besides the usually employed probe dipenylhexatriene (DPH) five new probes were tested. Single cell fluorescence polarization measurements on differentiating teratocarcinoma cells demonstrated an increase in fluorescence polarization in all cells with DPH and tetramethyl-diphenylhexatriene (TMA-DPH) . Since DPH penetrated the cells besides labelling the plasmamembrane it is impossible to attribute the increase in fluorescence polarization to a decrease in plasmamembrane fluidity, as has been found by others with a different technique (145). Staining of the plasmamembrane was more prominent with TMA-DPH and recent results in the literature (151) show that under certain conditions exclusive plasmamembrane labelling can be accomplished. Since our measurements have demonstrated the usefulnes of TMA-DPH as fluorescence polarization probe it should be possible with this probe to determine changes in the plasmamembrane exclusively. The increase in fluorescence polarization occurred as a late event after cell differentiation.

Cocultivation (appendix paper 5). We investigated whether teratocarcinoma cells could be induced to differentiate by long term cocultivation with differentiated mouse endoderm cells (which are the first differentiated cells appearing after teratocarcinoma cell differentiation). The DNA content of the endoderm cells was duplicated and the resulting difference in DNA content was exploited (by using the viable DNA stain Hoechst 33342) as basis for the selection of the teratocarcinoma cells after cocultivation. Analysis of the two dimensional protein pattern of the flow sorted cells revealed that cocultivation had not induced the teratocarcinoma cells to differentiate.

Cybridization. In the cybridization system it was investigated whether fusion with the cytoplasm of a differentiated mouse cell would be sufficient to trigger the teratocarcinoma cells to become permanently differentiated cells. The first step in the cybridization procedure is the isolation of enucleated cells (cytoplasts). By making use of the DNA stain Hoechst 33342 mentioned above, nearly pure (>99%) cytoplast preparations could be sorted from the mixture of cells, cytoplasts and karyoplasts (isolated nuclei) obtained after enucleation on Ficoll density gradients (appendix paper 1). This method was further improved by using a different gradient material (Percoll).It was also shown that cytoplasts containing fluorescent beads could be isolated this way (appendix paper 2).

Cytoplasts from differentiated mouse endoderm- and neuroblastoma cells containing green fluorescent beads were isolated and fused with unlabelled mouse teratocarcinoma cells. This resulted in a mixed population with only a small percentage of cybrids. By employing the Hoechst DNA stain, cybrids (cells with nuclear fluorescence and fluorescent beads) could be distinguished from unfused parental cells and cytoplasts. For this combination of fluorochromes two-laser excitation was necessary. The cybrids were sorted out one by one and directly cloned in separate culture vessels by employing the single cell sorting capability of the cell sorter. Biochemical analysis of the resulting colonies of proliferating cybrid cells revealed that they resembled the parental teratocarcinoma cells and therefore that no permanent induction of differentiation had occurred (appendix paper 4).

Hybridization. We studied gene expression of heterokaryons formed by fusion of mouse teratocarcinoma cells with primary human fibroblasts. Since only a small percentage of the cells in the mixture after fusion are heterokaryons, these cells have to be isolated before biochemical analysis can be performed.

A procedure developed in this laboratory for the isolation of hetrokaryons by flow sorting (40) was examined in more detail and extended. In a similar approach using red- and green fluorescent stearylamines, red-green fluorescent heterokaryons could be isolated (appendix paper 3).

Using the stearylamine labels, teratocarcinoma x fibroblast heterokaryons were isolated (appendix paper 7). Two-dimensional gelelectrophoresis of radiolabelled proteins showed that, after

fusion, no new proteins other than those already synthesized by the parental cells were expressed. There were however three proteins which were synthesized in larger amount then in either of the parental cells. For one of these proteins it is very likely that this reflects the enhanced synthesis of the mouse homolog of a protein expressed in large amounts by human fibroblasts and differentiated mouse cells. Moreover collagen type-I synthesis which is specific for the differentiated fibroblasts continued in the heterokaryons but activation of mouse type-I collagen could not be demonstrated. Therefore no activation of a previously completely silent mouse gene was found, but the fused cells phenotypically did resemble the differentiated fibroblasts rather then the undifferentiated teratocarcinoma cells.

Both the cybridization and cocultivation experiments described in this thesis have inherent limitations and can only demonstrate the presence of activating factors under certain conditions. Our finding with these cell systems of no induction of cell differentiation therefore does not necessarily mean that such factors do not exist. The heterokaryon system can give the most direct information, both about activation and extinction.

In teratocarcinoma heterokaryons the expression of differentiated gene products is not extinguished, contrary to what usually has been found after fusion of two differentiated cells. A necessary prerequisite for activation is therefore fulfilled. Although the teratocarcinoma heterokaryon system therefore has the potential to enable the study of activation of genes coding for differentiated gene products, still an additional stimulus appears to be necessary before activation can occur. This may be accomplished by fusion with more closely related or with tetraploid cells.

Samenvatting.

Zoogdieren zoals de mens en de muis bevatten een groot aantal verschillende typen cellen. Al deze verschillende soorten cellen zijn ontstaan uit één cel, de bevruchte eicel. Het proces waarbij stabiele verschillen tussen cellen ontstaan wordt cel differentiatie genoemd. Teratocarcinoma cellen hebben vele eigenschappen gemeen met nog ongedifferentieerde cellen uit het embryo (blastocyst stadium). Daar deze cellen *in vitro* gekweekt kunnen worden, waarbij ze het vermogen tot zowel *in vivo* als *in vitro* differentiatie behouden, vormen ze een goed model systeem voor het bestuderen van de vroege cel differentiatie.

De experimenten in dit proefschrift zijn gedaan met een zogenaamde "fluorescence activated cell sorter". Wanneer men een suspensie van cellen heeft kunnen met dit apparaat van iedere cel afzonderlijk bepaalde optische eigenschappen (meestal fluorescentie) gemeten worden. Bovendien kunnen cellen met specifieke optische eigenschappen uitgesorteerd worden. Cellen kunnen op verschillende manieren fluorescerend gemaakt worden door gebruik te maken van fluorescerende merkstoffen die verschillende cellulaire bestanddelen aankleuren. Bovendien zijn er wat de "cell sorter" betreft ook nog verschillende gebruiksmogelijkheden (bijvoorbeeld excitatie met twee lasers en het sorteren van slechts één cel per keer). De kombinatie van de verschillende keuzemogelijkheden maakt dat er een groot aantal verschillende toepassingsmogelijkheden zijn.

Een aantal aspecten van de *in vitro* differentiatie van teratocarcinoma cellen kunnen juist goed bestudeerd worden door de "cell sorter" toe te passen en daar gaat dit proefschrift over.

Het experimentele werk kan in vier delen onderverdeeld worden, fluorescentie polarisatie metingen, samenkweek-, cybridisatie- en hybridisatie experimenten.

Fluorescentie polarisatie (artikel 6, appendix). Muize teratocarcinoma cellen kunnen tot *in vitro* differentiatie aangezet worden door ze te behandelen met bepaalde chemische stoffen. Eén van de parameters die bij differentiatie verandert is de "vloeibaarheid" van de celmembraan. Deze verandering kan indirekt bepaald worden door de polarisatie van de fluorescentie te meten van fluorescerende merkstoffen die zich in de membraan bevinden.

Naast de veelal gebruikte merkstof diphenylhexatrieen (DPH) werden vijf nieuwe merkstoffen getest. Fluorescentie polarisatie metingen met de "cell sorter" aan differentierende teratocarcinoma cellen toonden aan dat er in alle cellen een toename in fluorescentie polarisatie was. De toename in fluorescentie polarisatie werd gevonden met DPH en tetrametyl-diphenylhexatrieen (TMA-DPH). Bij observatie met de fluorescentiemikroskoop bleek dat DPH naast de celmembraan ook binnen in de cel strukturen aankleurde. Hierdoor kan de toename in fluorescentie polarisatie niet éénduidig toegeschreven worden aan een afname in de "vloeibaarheid" van de plasmamembraan, zoals met behulp van een andere techniek door anderen gevonden is (145). De kleuring van de celmembraan was duidelijker

met TMA-DPH en recente resultaten in de litteratuur (151) laten zien dat met deze merkstof onder bepaalde inkubatie kondities wél uitsluitend de celmembraan gemerkt kan worden. Aangezien onze metingen hebben aangetoond dat TMA-DPH gebruikt kan worden voor fluorescentie polarisatie metingen moet het met deze kleurstof mogelijk zijn om uitsluitend veranderingen in de celmembraan te bepalen. De toename in fluorecentie polarisatie trad pas op geruime tijd nadat de cellen met differentiëren begonnen waren.

Samenkweek (artikel 5, appendix). Bij deze experimenten hebben we onderzocht of teratocarcinoma cellen tot differentiatie aangezet konden worden door ze samen te kweken met gedifferentieerde muize endoderm cellen. Deze endoderm cellen zijn de eerste gedifferentieerde cellen die verschijnen bij de differentiatie van teratocarcinoma cellen. Het DNA gehalte van de endoderm cellen werd verdubbeld en het zo ontstane verschil in DNA gehalte tussen de teratocarcinoma cellen en de endoderm cellen werd gebruikt om de teratocarcinoma cellen na samenkweek uit te sorteren. Hiertoe werd het DNA gekleurd met de vitale fluorescerende kleurstof Hoechst 33342. De analyse van het tweedimensionale gelelektroforese patroon van de eiwitten die door de gesorteerde cellen gesynthetiseerd werden, toonde aan dat het samenkweken met de endoderm cellen de teratocarcinoma cellen niet tot differentiatie aangezet had.

Cybridisatie. Met behulp van cybriden hebben we onderzocht of fusie met het cytoplasma van een gedifferentieerde cel voldoende zou zijn om de teratocarcinoma cellen blijvend te veranderen in gedifferentieerde cellen. De eerste stap bij de cybridisatie procedure is de isolatie van ontkernde cellen (cytoplasten). Door gebruik te maken van de Hoechst kleurstof konden met behulp van de "cell sorter" praktisch zuivere populaties cytoplasten (>99%) geïsoleerd worden uit het mengsel van cellen, cytoplasten en losse kernen dat na ontkerning op een Ficoll gradient verkregen werd (artikel 1, appendix). Deze methode werd nog verbeterd door gebruik te maken van een ander gradient materiaal (Percoll). Bovendien werd aangetoond dat op deze wijze ook cytoplasten die fluorescerende latex bolletjes bevatten geïsoleerd konden worden (artikel 2, appendix).

Cytoplasten van gedifferentieerde muize endoderm- en neuroblastoma cellen die groen fluorescerende bolletjes bevatten werden geisoleerd en gefuseerd met teratocarcinoma cellen. Dit leidde tot een klein percentage cybriden in het fusie mengsel. Door weer de Hoechst kleurstof te gebruiken konden cybriden (cellen met fluorescerende kernen en bollen) onderscheiden worden van ongefuseerde cellen en cytoplasten. Deze kombinatie van fluorescerende stoffen vereiste excitatie met twee lassers. De cybriden werden één voor één uitgesorteerd en gekloneerd door gebruik te maken van de mogelijkheid voor het sorteren van één cel per keer. De analyse van de resulterende kolonies van prolifererende cybride cellen liet zien dat ze leken op teratocarcinoma cellen en dat derhalve geen induktie van differentiatie was opgetreden (artikel 4, appendix).

Hybridisatie. In deze experimenten hebben we heterokaryonten onderzocht, gemaakt door fusie van teratocarcinoma cellen met menselijke fibroblasten. Daar slechts een gering percentage van de

cellen fuseerde moesten de heterokaryonten gezuiverd worden voordat biochemische analyse kon plaatsvinden.

Een in dit laboratorium ontwikkelde methode voor de isolatie van heterokaryonten met behulp van de "cell sorter" (40) werd nader onderzocht en uitgebreid. Ook door gebruik te maken van rood- en groen fluorescerende membraanmerkstoffen konden rood-groen gekleurde vitale heterokaryonten uitgesorteerd worden (arikel 3, appendix).

Deze membraankleurstoffen werden vervolgens gebruikt voor de isolatie van teratocarcinoma x fibroblast heterokaryonten (arikel 7, appendix). Tweedimensionale gelelektroforese van eiwitten gesynthetiseerd door de heterokaryonten (en door synkaryonten die hieruit gevormd werden) liet zien dat geen andere eiwitten tot expressie werden gebracht dan welke al door de ouder cellen gesynthetiseerd werden. Drie van deze eiwitten werden echter in grotere hoeveelheden gesynthethiseerd dan in de ouder cellen. Voor één van deze eiwitten is het waarschijnlijk dat het de muize homoloog is van een eiwit dat in fibroblasten van de mens veel voorkomt. Bovendien komt het veel voor in gedifferentieerde muize cellen. De synthese van collageen type I, specifiek voor gedifferentierde fibroblasten, bleef doorgaan na fusie maar aktivatie van het muize type I collageen kon niet worden aangetoond.

In de gefuseerde cellen werd derhalve geen aktivatie van een tevoren niet tot expressie gebracht muize gen gevonden, maar wel leken de cellen phenotypisch op de gedifferentieerde fibroblasten en niet op de ongedifferentieerde teratocarcinoma cellen.

Zowel de cybridisatie als de cocultivatie experimenten die in dit proefschrift beschreven zijn hebben hun beperkingen en kunnen de aanwezigheid van aktiverende faktoren slechts onder bepaalde voorwaarden aantonen. Dat wij geen induktie van differentiatie gevonden hebben met deze experimenten hoeft niet te betekenen dat deze faktoren ook niet bestaan.

In teratocarcinoma heterokaryonten wordt de expressie van gedifferentieerde gen produkten van de gedifferentieerde oudercel niet onderdrukt, in tegenstelling tot wat in het algemeen bij fusie van twee gedifferentieerde cellen gevonden is. Aan een noodzakelijke voorwaarde voor het optreden van aktivatie wordt derhalve voldaan. Hoewel het teratocarcinoma heterokaryonten systeem dus mogelijkheden biedt voor de bestudering van aktivatie van genen die voor gedifferentieerde produkten koderen blijkt toch nog een extra stimulans nodig te zijn voordat aktivatie optreedt. Dit kan misschien worden bereikt door te fuseren met cellen die dichterbij de teratocarcinoma cellen staan voor wat betreft hun differentiatie stadium of met cellen die tetraploid zijn (toepassing van het gen-dosis effect).

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Curriculum vitae.

De schrijver van dit proefschrift werd op 7 maart 1953 te Vlaardingen geboren. Na de lagere school behaalde hij in 1971 het einddiploma Gymnasium β aan het Groen van Prinsterer Lyceum te Vlaardingen.

In 1971 begon hij met de studie scheikunde aan de Rijks Universiteit te Leiden alwaar hij in 1974 het kandidaatsexamen (S2) behaalde. In januari 1978 werd het doktoraalexamen afgelegd. (Hoofdvak biochemie bij Prof.Dr.L.Bosch en bijvakken: biofysica bij Dr.J.Amesz en fysische chemie bij Dr.H.W.Joustra, plus onderwijsbevoegdheid scheikunde).

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Van september 1983 tot september 1984 studeerde hij procestechnologie aan de Technische Hogeschool te Delft.

Sinds 17 september 1984 is hij in dienst van Organon International B.V. te Oss.

Hij is getrouwd en heeft één zoon en twee dochters.

PAPER 1

Isolation of anucleate cells using a fluorescence activated cell sorter (FACS II)

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Summary. Human fibroblasts or mouse teratocarcinoma cells were enucleated by density gradient centrifugation in the presence of cytochalasin B (CB). The resulting mixed population of nucleated and anucleate cells was further purified by flow sorting, using the dye Hoechst 33342 as a fluorescent label for the nucleated cells. The purity of the anucleate cells obtained with this technique was at least 99%, as was shown by histological staining of the sorted fractions. Sorted enucleated fibroblasts were shown to have an intact cell membrane as indicated by their ability to convert fluorescein diacetate into fluorescein and to accumulate this product. They were found to attach and spread when cultured and showed protein synthesis immediately after enucleation, evidenced by the incorporation of [^{3}H]leucine. Sorted enucleated teratocarcinoma cells also had an intact cell membrane, but they did not attach when cultured.

Anucleate cells (cytoplasts) can be used to study the influence of cytoplasmic factors of one cell type on processes in another cell type. For this purpose they are fused with whole cells forming cytoplasmic hybrids (cybrids) [1] or with minicells (karyoplasts) forming a reconstituted cell [2, 3].

Although spontaneous enucleation of cells can be induced by cytochalasin B (CB) [4], a combination of a centrifugational force and CB treatment increases the yield of anucleate cells, see for review [5]. Some of the methods developed [6, 7, 8] require that the cells are attached to a substratum while others, using density gradient centrifugation, offer the possibility to enucleate cells in suspension [9, 10]. A nearly pure anucleate cell fraction of mouse L cells has been obtained by one of these last methods [9]. Results obtained by Bossart et al. [10] with human HEp-2 cells and a different gradient material showed however that a further purification of the anucleate cell fraction, using a second centrifugation step, was necessary. In our hands the contamination of nucleated cells in the anucleate cell fraction was considerable. We have purified this fraction further by making use of the difference in DNA content between nucleated and anucleate cells.

The vital fluorescent DNA stain Hoechst 33342 [11, 12] was used to stain the mixed population of anucleate and nucleated cells, and by subsequent flow sorting with a fluorescence activated cell sorter (FACS II) [13, 14] the anucleate cell fraction was sorted out.

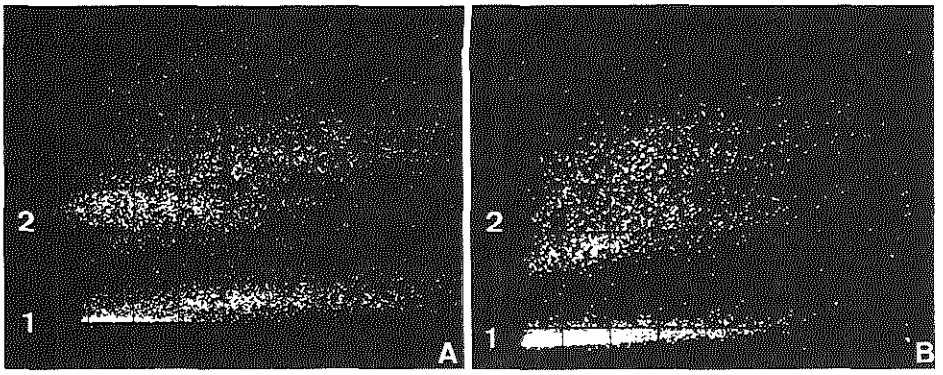

Fig. 1. Abscissa: scatter intensity/cell; *ordinate:* fluorescence intensity/cell. (*a*) Fibroblasts (4000 cells); (*b*) PCC4 cells (4000 cells).

Flow analysis of the cell population after enucleation by density gradient centrifugation in the presence of CB. Cells were labelled with Hoechst 33342, at 37°C for 30 min.

In this communication we describe the purification of anucleate cells from cultivated human fibroblasts and mouse teratocarcinoma cells by means of this technique.

Materials and Methods

Cells and media. Human skin fibroblasts and a mouse teratocarcinoma cell line, PCC4 Aza 1 [15] (a kind gift from Dr T. Boon, Unité de Génétique Cellulaire, Int. Inst. of Cellular and Molecular Biology, Brussels), were cultured in Ham's F10 medium supplemented with 15% fetal calf serum (FCS), penicillin (100 U/ml) and streptomycin (0.1 mg/ml).

Enucleation and DNA staining. Cells were enucleated using a Ficoll gradient [9]. Solutions of the appropriate concentrations Ficoll 400 (Pharmacia) (% w/w) were prepared by dissolving previously autoclaved Ficoll in Ham's F10 medium buffered with HEPES, PIPES and BES (BDH) (10 mM of each), pH 7.2. Cytochalasin B (CB) (Serva) was dissolved in DMSO (1 mg/ml) and added to the Ficoll solutions till an end concentration of 10 μg/ml (fibroblasts) or 5 μg/ml (PCC4). The following discontinuous gradients were prepared in 13 ml centrifuge tubes (SW 41, polyallomer); fibroblasts: 1 ml 33%, 1.5 ml 28%, 1.5 ml 23%, 1 ml 17%, 1.5 ml 13%, 1 ml 9%; PCC4: 1.5 ml 33%, 1.5 ml 13%, 1.5 ml 11%, 1.5 ml 10% and 1.5 ml 7.5%. Gradients were preincubated for at least 1 h at 37°C. Cells were harvested with trypsin (fibroblasts) or trypsin/EDTA (PCC4), suspended in medium with serum, washed twice with medium and finally resuspended in 2 ml 9% (fibroblasts) or 1.5 ml 7.5% (PCC4) Ficoll+CB. Fibroblasts were preincubated for 15 min in this solution. The cell suspension was layered on top of the gradient and covered with medium. Centrifugation was done at 37°C for 1 h at 25000 rpm in a Beckman L5-65 ultracentrifuge (rotor SW41, g_{av}=77000 g). After centrifugation the appropriate fractions were collected, diluted with medium, washed twice, and incubated with Hoechst 33342 (10 μM) (generously provided by Dr J. W. M. Visser, TNO, Rijswijk, and Dr H. Loewe, Hoechst AG, Frankfurt) for 30 min at 37°C. Thereafter the cells were cooled to 4°C and kept at that temperature during the sorting procedure.

Cell sorting. Cell sorting was performed with a FACS II cell sorter (Becton & Dickinson) equipped with a 5 W argon ion laser (Spectra Physics, 164-05) and barrier filters KV 400 and K 445 (Schott). Laserlight at 351 to 364 nm was used at a constant output of 35 mW. Cells were sorted directly on coverslips which were placed in Petri dishes, using a 60 μm nozzle. To test the purity of the sorted samples the droplet containing the sorted cells was covered with the same volume of fixative (ethanol : acetic acid = 1 : 1) and the coverslip was left to dry. Cell staining was done with lacto-aceto orcein [16] for 10 min. Also sorted nucleated and anucleate cells which had been cultured overnight were fixed with Bouin's fixative and stained with haematoxylin-eosin.

Vitality tests. (*a*) Sorted anucleate cells were incubated with fluorescein diacetate [17, 18] (0.1 μg/ml). After 5 min incubation at room temperature, cytoplasts were examined for fluorescein accumulation using a fluorescence microscope (Leitz, diavert with epi-illumination).

(*b*). Anucleate fibroblasts were sorted, and cultured for 16 h in the presence of [^{3}H]leucine (10 μCi/ml; spec. act. 49 Ci/mmol; Radiochemical Centre, Amersham). After a 2 h chase they were fixed and autoradiography was performed, using Ilford K2 emulsion.

Results

Fibroblasts. After centrifugation two major bands were observed at the 28/23 and 23/

Fig. 2. Sorted and attached enucleated fibroblasts, cultured overnight and stained with H/E.

17% Ficoll interfaces, both containing a mixed population of anucleate and nucleated cells (±50% enucleated cells in both fractions), as shown by inspection of H/E stained preparations of these fractions cultured overnight. Another band at the 9/0% interface was found to consist of cell debris only. The two major bands were pooled, washed and used in the subsequent sorting step. After vital staining with Hoechst 33342 this population was analysed with the FACS II cell sorter (fig. 1*a*). Two separated subpopulations could be observed, one which showed only a weak fluorescence "1" and another showing a strong fluorescence "2". The subpopulations were sorted out and stained with lacto-aceto orcein. Inspection of the stained preparations showed that subpopulation 2 contained cells with nuclei. Subpopulation 1, on the other hand, consisted almost entirely of anucleate cells. The purity of this sorted subpopulation was better than 99%. Sorted anucleate cells were found to attach and spread when cultured (fig. 2). Inspection of H/E-stained preparations of these cells showed that less than 1% of the attached cells contained a nucleus.

The capacity of the sorted anucleate cells to adhere and spread when cultured, gives an indication that these cytoplasts remain intact to a large extent. Two further tests were performed with regard to this point. First the ability of the sorted anucleate cells was tested to convert fluoresceindiacetate (FDA) into fluorescein and to accumulate this product. More than 90% of the anucleate cells showed fluorescein fluorescence immediately after sorting, indicating an intact cell membrane. Secondly, autoradiography of sorted anucleate cells, cultured in the presence of [^{3}H]leucine, showed incorporation of radioactivity in all attached cells. This indicates that after enucleation, staining and sorting protein synthesis in the cytoplasts is still possible.

PCC4 cells. After centrifugation of the teratocarcinoma cells one band was found at the 7.5/0% Ficoll interface which contained mainly cell debris. All other bands were pooled, washed free of Ficoll and incubated with Hoechst 33342. The percentage of enucleated cells was about 50% (determined with fluorescence microscopy). Analysis with the cell sorter showed two separated subpopulations "1" and "2" (fig. 1*b*). Staining with lacto-aceto orcein of the sorted anucleate cells (subpopulation 1) showed that the contamination with nucleated cells was 1%. The structural integrity of the sorted anucleate cells was tested with FDA, and 95% of the cytoplasts were found to be intact. After culturing overnight the cytoplasts did not attach to the Petri dish.

Discussion

Several methods for the enucleation of cells have been described. The density

gradient techniques [9, 10] in principle offer the possibility to enucleate cells that adhere insufficiently or not at all. A further advantage is that large numbers of cells ($\pm 10^8$) can be handled. The purity of the anucleate cell fractions thus obtained, however, may not always be sufficient. For example, when using cytoplasts in the study of the influence of cytoplasmic factors on gene expression, a too high percentage of contaminating nucleated cells makes a correct interpretation of the results impossible [19]. In general it is difficult to obtain pure anucleate cell fractions directly in the enucleation gradient. This is especially true for cultured fibroblasts and teratocarcinoma cells that appear not to be homogeneous in density (H. A. de Wit-Verbeek, pers. commun.).

We designed the gradient for the teratocarcinoma cells in attempts to obtain a pure anucleate cell fraction directly in the enucleation gradient. The one used for the fibroblasts was originally designed for another cell type (villus cells from rat jejunum) but was found useful for fibroblasts as well. With various CB concentrations tested (5–30 μg/ml), no large differences were found in percentages of enucleated cells, and 5 or 10 μg/ml was used for our enucleation procedure. The fluorescent DNA stain Hoechst 33342 was employed because staining is very fast, and the dye is lost again when the cells are cultured in its absence [12], which is important when the cytoplasts are to be fused with other cells.

The results we have obtained show that it is possible to purify anucleate cells from a mixed population by flow sorting. The purity of the anucleate cell fraction was at least 99%. This figure compares favourably with purities obtained by other methods [2, 6, 7, 8, 10]. The number of anucleate cells that can be obtained with our procedure is limited by the working speed of the cell sorter, and is dependent on the percentage of anucleate cells in the population to be sorted. In our experiments it took about 40 min to sort 10^6 anucleate cells.

The cell membrane of sorted enucleated fibroblasts is structurally intact and the cytoplasts attach and spread when cultured. The enucleated teratocarcinoma cells also had an intact cell membrane, but they did not attach when cultured. This was also the case for sorted nucleated teratocarcinoma cells and for the not-sorted mixed population. We found that centrifugation of teratocarcinoma cells through a Ficoll gradient, even in the absence of CB, greatly reduces their ability to attach and to proliferate (res. not shown). It seems that fibroblasts and teratocarcinoma cells differ in their ability to survive the mechanical forces during gradient centrifugation. Also the cell sorting procedure itself may impose some stress on the cells. Nevertheless we think the method described is useful for obtaining pure cytoplasts, not only from cell types that do not adhere but also from cell types that do adhere to a substratum.

Cytoplasmic hybrids (cybrids) formed by the fusion of anucleate cells with other cells can be selected from a fusion mixture by selective media [20]. Other studies describe the use of latex spheres in order to recognize the fusion product shortly after fusion [2, 3, 21]. We have shown, that flow sorting of heterokaryons is possible using different types of fluorescing latex beads as a cytoplasmic marker for the parental cell types [22]. The combination of this method and the technique reported here for the isolation of cytoplasts will probably make it possible to obtain pure cybrids, without the use of selective media.

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PAPER 2

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SEPARATION OF CYTOPLASTS FROM NUCLEATED CELLS BY FLOW SORTING USING HOECHST 33342

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Mouse teratocarcinoma cells were enucleated on Percoll density gradients containing cytochalasin B. Cytoplasts were purified from the resulting mixture of nuclei, enucleated cells, and complete cells by flow sorting using the vital DNA stain Hoechst 33342. The sorted cytoplasts had an intact cell membrane, as shown by their ability to convert fluorescein-diacetate into fluorescein and to accumulate this product. The cytoplasts attached when cultured.

Cytoplasts from human skin fibroblasts were prepared using the same procedure. These cytoplasts also had an intact cell membrane, and attached and spread when cultured. Protein synthesis was still present as shown by the incorporation of ^{3}H-leucine. Fibroblasts labelled with fluorescent latex microspheres were also enucleated and cytoplasts were purified by flow sorting using the signals of both the green fluorescent beads and the Hoechst dye.

The effect of staining with Hoechst followed by flow sorting, on the growth of human skin fibroblasts was investigated.

Key words: Cytoplasts; flow sorting; Hoechst 33342.

Cytoplasts (anucleate cells) can be used to study the influence of cytoplasmic factors of one cell type on processes in another cell type. For this purpose they can be fused with other cells, forming cybrids, or with nuclei (minicells), forming a reconstituted cell.

Several methods of obtaining cytoplasts have been developed, see for review (9). Cytochalasin B (cyt B) is used to break down the microfilaments, whereafter, under a centrifugational force, the nucleus is expelled from the cell. Some methods require that the cells be attached to a substratum, thus excluding cell types that do not or not sufficiently adhere. Enucleation by density gradient centrifugation (2, 13) on the other hand, is in principle applicable to all cell types. In most cases a pure cytoplast preparation cannot be obtained directly in this way. Methods of purifying a mixture of cytoplasts and nucleated cells, by low speed density gradient centrifugation, have been reported (2, 6). We have recently shown that it is possible to purify cytoplasts from an enucleation mixture, obtained by centrifugation on *Ficoll* density gradients, to a purity of 99% or more by flow sorting (11). In these isolations, the vital DNA stain Hoechst 33342 (1) was used to discriminate between anucleate and nucleated cells.

In this report we describe the use of *Percoll* gradients for enucleating mouse teratocarcinoma cells and human skin fibroblasts followed by flow sorting, to purify the cytoplasts from the mixed populations. Furthermore, we investigated the enucleation and purification of fibroblasts labelled with fluorescent latex spheres, to produce fluorescent cytoplasts. Finally we studied the effect of staining and sorting on the growth of fibroblasts.

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MATERIALS AND METHODS

Cell culture

Human skin fibroblasts and mouse teratocarcinoma cells (PCC4 AZA 1) (4) were cultured in HAM's

F10 medium, supplemented with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 µg/ml). Cells to be labelled with green fluorescent latex spheres (*Polysciences*, 1.83 µm, cat. no. 9847, lot no. 2-2426) were incubated for at least 24 hours in medium containing 7×10^6 beads/ml. For growth experiments, 50,000 fibroblasts were seeded in 3.5 cm plastic culture dishes. After one week of culture, cells were trypsinized and washed, and protein was determined according to (7).

Enucleation procedure

A gradient solution of 40% v/v (density 1.058g/cm^3) Percoll *(Pharmacia)* in F10 containing 5 µg/ml cytochalasin B (*Serva*) (stock solution 1 mg/ml in DMSO) was prepared. Cells were harvested with trypsin (fibroblasts) or trypsin/EDTA (PCC4), washed once with F10, resuspended in 1 ml gradient solution and preincubated for 30 minutes at 37°C. SW41 polyallomer centrifuge tubes were filled with 8.5 ml gradient solution (37°C) and the cell suspension was layered on top. Centrifugation was performed using a prewarmed SW41 rotor, at 37°C, 22.500 rpm (64.000 g_{av}) for 20 minutes in a Beckman L5-65 ultracentrifuge. After centrifugation, the appropriate cell fraction was diluted five times with F10, centrifuged and washed once with F10.

Cell staining and sorting

Cells were stained with Hoechst 33342 (10µM, 30 minutes, 37°C) and sorted as described previously (11) using a FACS II cell sorter *(Becton and Dickinson)*. For flow sorting of cytoplasts labelled with green fluorescent latex spheres, the filter combination was adjusted. A KV399 filter was used to block scattered laser light. The fluorescent light was divided by a dichroic mirror (RKP506), which passes on wavelengths longer than 506 nm and reflects shorter wavelengths. In front of the «blue» photomultiplier tube (PMT) KP490 and K445 filters were inserted, while K510 and K515 filters were placed before the «green» PMT (All filters were from *Schott* except RKP506 which was from *Balzers)*.

Viability tests

To test the intactness of the cell membrane, sorted cytoplasts were incubated for 5 minutes at room temperature with fluoresceindiacetate (10) (0.1 µg/ml), and then examined for fluorescein accumulation using a fluorescence microscope (*Leitz*, diavert with epi-illumination). Residual protein synthesis was tested with enucleated fibroblasts. Sorted cytoplasts were allowed to attach for 1.5 hours and then incubated with ^{3}H-leucine (10 µCi/ml, spec. act. 54Ci/mmol, *Radiochemical Centre Amersham)*. As a control 20 µg/ml cycloheximide *(Boehringer)* was added together with the ^{3}H-leucine. After 1 hour, cells were fixed with Bouin's fixative and autoradiography was performed, using Ilford K2 emulsion.

RESULTS

Survival of cells after staining with Hoechst 33342 and sorting

Fibroblasts were stained with Hoechst 33342 and sorted under the same conditions as used for cytoplast purification. Duplicate cultures of sorted cells were grown for 7 days to assay the influence of staining and sorting on cell growth. At the end of the culture period, the growth of stained and sorted cells was greatly inhibited if the medium had not been changed (Table 1). When the medium was changed daily after staining and sorting, the growth of the cells did not differ from that of control cells.

Table 1. Influence of Hoechst 33342 and sorting on growth of fibroblasts.

	change of medium[a]	percentage growth after 7 days[b]
control cells	+	1 509
	–	1 280
stained and sorted cells	+	1 532
	–	180

a. In some of the dishes the medium was not changed (–) and in others it was changed daily (+).
b. The protein content per dish was measured. Results are expressed as percentage increase in protein relative to the input at day zero.

Enucleation of teratocarcinoma cells on Percoll gradients

Enucleation of teratocarcinoma cells resulted in two bands, one at the bottom and one in the upper part of the gradient. In between, little cellular material was present. After washing, the material in the bands was stained with Hoechst 33342. Examination with the fluorescence microscope showed that the upper band consisted mainly of cell debris. The lower band, however, contained a mixture of cytoplasts and nucleated cell components. This mixture was analysed with the cell sorter (Fig. 1a). Two distinct subpopulations were observed. Subpopulation 1 consisted of cyto-

plasts and subpopulation 2 of nucleated cell components (shown by fluorescence microscopy of sorted cells). Cytoplasts (about 20% of all signals) were sorted out and incubated with FDA. Fluorescein fluorescence, indicating an intact cell membrane, was observed in all the cytoplasts. A large number of the sorted cytoplasts attached when cultured under tissue culture conditions, but only a minority also spread.

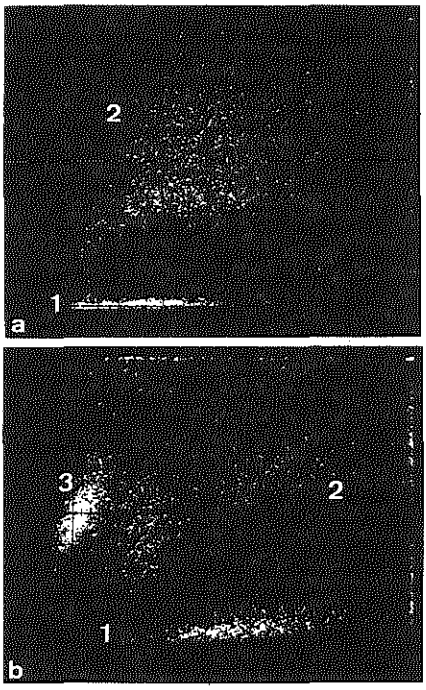

Fig. 1. Flow analysis of the cell population after enucleation by density gradient centrifugation on Percoll gradients in the presence of cytochalasin B. Cells were labelled with Hoechst 33342, at 37°C for 30 minutes.
Abscissa: scatter intensity per cell; *ordinate:* fluorescence intensity per cell.
a) teratocarcinoma cells (4000 cells)
b) fibroblasts (4000 cells).

Isolation of enucleated fibroblasts

The same enucleation procedure was also applied to fibroblasts, and identical results were obtained after centrifugation. The band at the bottom of the gradient was used for flow sorting. In this case three subpopulations were observed (Fig. 1b): subpopulation 1, consisting of cytoplasts, subpopulation 2, containing complete cells, and subpopulation 3 having isolated nuclei. Cytoplasts (about 30% of all signals) were sorted out. All cytoplasts had an intact cell membrane, and when cultured they attached and spread. Incorporation of ^{3}H-leucine was observed in 90% of the sorted cytoplasts (Fig. 2), and addition of cycloheximide reduced grain numbers over the cytoplasts to background level. This indicates that protein synthesis still takes place in the cytoplasts after flow sorting.

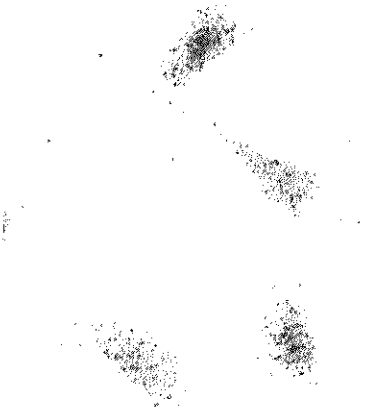

Fig. 2. Autoradiograph of sorted and attached enucleated fibroblasts, cultured for 2.5 hours after sorting and then incubated with ^{3}H-leucine for 1 hour: Stained with H/E.

Fibroblasts labelled with fluorescent latex microspheres were also enucleated. Analysis as described for unlabelled cells resulted in suboptimal separation of the subpopulations. Therefore cells were analysed using both the signals of the blue Hoechst fluorescence and the green fluorescence of the microspheres (Fig. 3). Cytoplasts, containing only latex beads, were now observed as a separate subpopulation (1, Fig. 3) and could be sorted out. The cytoplasts attached when cultured and for a large part also spread.

DISCUSSION

Enucleation of cells in suspension has the advantage that in principle all cell types can

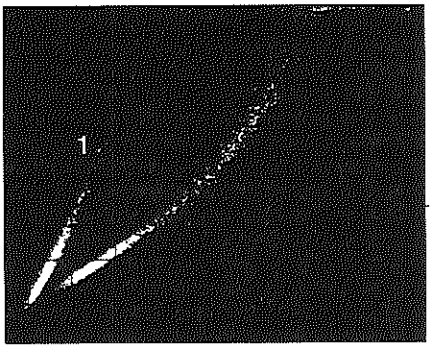

Fig. 3. Enucleation of fibroblasts labelled with green fluorescent microspheres. Flow analysis (4000 cells) of the cell population after enucleation. Cells were stained with Hoechst 33342 as in fig. 1. *Abscissa*: Hoechst fluorescence intensity per cell; *Ordinate*: green fluorescence intensity per cell.

be enucleated. Furthermore, relatively large quantities of anucleate cells can be obtained. Except for cases where enucleation efficiency is almost 100%, as was reported for mouse L-cells (13), enucleation will however result in a mixture of nuclei, cytoplasts and whole cells. We have described the purification of such a mixture, obtained on density gradients of Ficoll, by flow sorting (11). In these experiments we found that sorted enucleated mouse teratocarcinoma cells did not attach when cultured, although most did have an intact cell membrane. As we found that centrifugation of these cells through Ficoll gradients without cyt B also greatly reduced their ability to proliferate, we have now investigated another gradient material, colloidal silica, which has also been used for the enucleation of cells (2). We have used the commercially available material, Percoll, which consists of colloidal silica coated with polyvinylpyrrolidone, has a very low osmotic value (< 20 mOsm) and is not toxic for cells (8).

Preliminary experiments (results not shown) showed that teratocarcinoma cells did survive centrifugation through Percoll gradients without cyt B, and when using the gradients to enucleate these cells, we found that sorted cytoplasts did attach when cultured. Possibly the elevated osmotic value of concentrated Ficoll solutions has been the cause for the reduced «viability» observed in the case of teratocarcinoma cells. The enucleation procedure with Percoll is much shorter and simpler and can also be applied to fibroblasts. Cytoplasts from these cells obtained after sorting were just as «viable», according to the criteria we applied, as the ones obtained with Ficoll gradients in earlier experiments (11). Since quantitative aspects were much the same as with Ficoll (about 10^6 cytoplasts could be sorted out in 30 to 40 minutes), we think that Percoll is a better choice for the gradient material.

The use of labelling of cells with microspheres for the identification of fusion products has been described earlier (3, 5, 12). Fluorescent labelled cytoplasts, obtained by density gradient enucleation, could also be purified by flow sorting. After fusion of these labelled cytoplasts with whole cells it is possible to identify the cybrids in the fusion mixture.

It is essential that the Hoechst dye is not toxic, because cytoplasts will probably transfer some of it to the nucleated partner in a fusion product. The growth experiments confirm an earlier report by others (1) and show that for isolation of viable fusion products it will be necessary to wash away the dye by changing the medium frequently.

Purification by flow sorting has the advantage that it is applicable to all cell types, whereas with the more conventional technique of density gradient centrifugation (2, 6) different gradients have to be constructed for different cell types. Together with the use of Percoll gradients, this method of purification will probably allow the isolation of cytoplasts from many different cell types.

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PAPER 3

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FLOW SORTING IN STUDIES ON METABOLIC AND GENETIC INTERACTION BETWEEN HUMAN FIBROBLASTS

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For the study of intra- and intercellular interactions between cultured human fibroblasts, balanced fluorescent cell markers were developed which allow flow-cytophotometric identification and flow sorting of cell populations. Using these aspecific cell labels, it was possible to isolate heterofluorescent heterokaryons by two-colour flow sorting (FACS II) after fusion of differently labelled parent populations. On the enriched heterokaryon fraction, complementation analysis of ß-galactosidase deficient variants could be performed.

Aspecific labelling and subsequent flow sorting can also be used to study intercellular interactions during cocultivation of human fibroblasts.

Key words: Flow sorting; heterokaryons; complementation; cocultivation.

During recent years much progress has been made in studies on the relation between clinical, biochemical and genetic heterogeneity in inborn errors of metabolism. For an increasing number of genetic diseases, it became apparent that «the same» enzyme defect may result in very different clinical features. To study the genetic background of this heterogeneity, somatic cell hybridization techniques have been used (2, 4, 5, 18, 25).

No selection procedures are available for heterokaryons. Complementation studies to measure restoration of enzyme activity must therefore be performed on homogenates of a mixed cell population containing non-fused parental cells, fused homokaryons and a varying percentage of heterokaryons. An alternative approach is the ultramicrochemical enzyme assay on single binuclear cells, but this is possible only for enzymes with an activity high enough to be measured by microfluorometry (6, 13). If methods of separation of larger quantities of heterokaryon material – flow sorting for instance – were available, this would stimulate further studies on genetic heterogeneity.

Until recently the application of flow sorting in cell biology has been primarily directed to the isolation of «pure» populations of cells from organs or tissue culture (for reviews see 1, 12). *Cell type specific* properties have been used in most of these isolations to separate the desired subpopulations from contaminating cells (10, 20). However, for the isolation of heterokaryons by flow sorting, the parent populations are not sufficiently different and therefore they were labelled with *aspecific* fluorescent markers (14), so that after cell fusion, heterokaryons could be isolated by two-colour cell sorting, and complementation analysis could be performed on enriched populations of heterokaryons. Complementation can be further unravelled by studies on cybrids *i.e.* fusions of whole cells of one type with enucleated cells from another mutant (3). For this type of study, flow sorting can be used both for the isolation of a pure population of cytoplasts (22, 23) and for the isolation of cybrids.

Many investigations have been carried out on the mechanism of uptake of exogenous

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enzyme by *in vitro* cultured enzyme deficient human fibroblast (15, 19, 26). So far, most model studies have been performed by administering purified enzyme from different sources to human mutant cells. A more physiological approach is the cocultivation of normal cells with enzyme deficient cells, followed by assays on both cell types to measure whether enzyme is being secreted by normal cells and can subsequently be taken up by deficient cells (8, 21). Again a serious limitation is the lack of selection methods, and as stated before, single cell analysis can only be performed in certain instances (21). Aspecific labelling of the parent populations followed by two-colour flow sorting after cocultivation would be a powerful technique in the study of intercellular exchange.

ASPECIFIC LABELLING OF FIBROBLASTS

When fluoresceine isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC) are excited at 488 nm the emission spectra overlap considerably. Using equal amounts of FITC and TRITC the contribution of the FITC emission at the wavelength for the detection of TRITC will be 9x as large as the TRITC signal itself. However, the contribution of the TRITC signal at the FITC emission peak is negligible (Fig. 1). This phenomenon of spectral crosstalk between different fluorescent dyes, excited at one laser-line presents serious difficulties for two-colour flow sorting using the optics of the FACS II machine. Several methods have been proposed to minimize the effective spectral crosstalk, for instance the use of two-colour excitation (24) or the application of stains that show the phenomenon of resonance energy transfer (16).

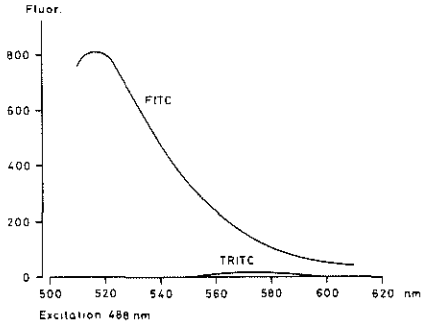

Fig. 1. Uncorrected emission spectra of FITC and TRITC (10^{-3}mg/ml) using 488 nm excitation.

In our system for aspecific labelling of cells, we tried to balance the emission of both fluorescent labels in such a way that the labelled cells were nearly equal in fluorescence at their respective emission peaks using 488 nm as the excitation wavelength. In addition, the filter system of the FACS was modified to obtain optimum rhodamine fluorescence without appreciable interference from the fluoresceine label. To investigate the optimum combination of fluorescent cell markers for two-colour flow sorting, we labelled

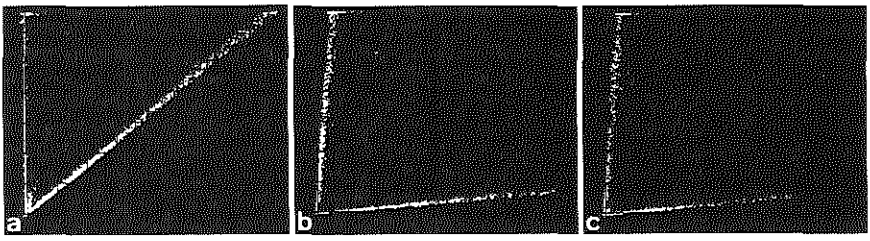

Fig. 2. Dot-plots of green- (x-axis) vs. red fluorescence (y-axis) for two mixed populations of fluorescent fibroblasts (red, green). 4000 cells.
All red-fluorescing fibroblasts were labelled with 1.6 µm red-fluorescing latex-spheres (Polysciences no. 7769; lot 2-1441).
a) green fibroblasts labelled with 1.83 µm green fluorescing beads (Polysciences no. 9847; lot 2-2426).
b) green fibroblasts labelled with 0.78 µm green fluorescing beads (Polysciences no. 7766; lot 2411).
c) green fibroblasts labelled with 0.52 µm FITC-NH_2 beads (14).

the fibroblasts with fluorescent latex beads (14). One population of cells was incubated with red fluorescent microspheres and another with batches of green fluorescent microspheres with different fluorescent intensities. The two-colour analysis with the FACS II illustrates that optimum separations of populations without appreciable fluorescent cross-talk could only be achieved when relatively weak fluorescing green microspheres were used (Fig. 2a as compared with Fig. 2b, c). When using FITC- and TRITC-stearylamine, as aspecific membrane markers (16), optimum separation between the green- and red-labelled cell populations was likewise only possible with low FITC-label concentration in comparison with the TRITC-stearylamine.

The influence of fluorescent labelling, with or without subsequent flow sorting, on the growth characteristics of fibroblasts was determined by protein determinations (17) in cultures of exponentially growing cells (11). The results indicate (Table 1) that labelling did impair the cell growth by 50% as compared with non-labelled cells. Additional flow sorting did not have an effect on the cell growth. Whether this decreased protein synthesis after labelling is due to an increased cell death or a decreased proliferation rate cannot yet be decided upon.

Table 1. Growth of fibroblasts after fluorescent labelling and flow sorting[a].

	Un-labelled	Latex		Stearylamine	
		Green	Red	Green	Red
Not sorted	610[b]	330	310	270	420
Flow-sorted	490	330	350	310	330

a. The growth was determined by comparing the protein content of a sample of treated cells (ca. 50 000 cells) with that of a 7 day culture of an equal sample.
b. Mean protein content of 7-day duplicate cultures as a percentage of initial samples.

FLOW SORTING AFTER CELL HYBRIDIZATION

After P.E.G. induced cell fusion (7) of cells labelled with red- or green-fluorescent microspheres, about 10% of heterofluorescent cells are found in addition to both parental strains (Fig. 3b as compared with Fig. 3a). This population of heterofluorescent cells contained 85% bi- and multinucleated cells after sorting (Table 2). Additional autoradiography after ^{3}H-thymidine prelabelling of one of the parent populations indicated that all the binucleated cells in the sorted fraction were in fact heterokaryons (14). This means that the percentage of heterokaryons in the fusion population can be raised from ca. 10% to 85% by two-colour flow sorting using fluorescent latex spheres as cell markers.

When stearylamine was used as an aspecific cell label (16), the separation between the parent population was sufficient to allow isolation of the heterofluorescent population by flow sorting (Fig. 3c, d). Seven hours after Sendai-virus-induced fusion (9) this heterofluorescent population contained 94% of bi- or multinucleated cells (Table 2). Again autoradiography indicated that the sorted binucleated cells were heterokaryons. So the percentage of heterokaryons can also be raised by using stearylamine as an aspecific cell label.

For complementation analysis we fused fibroblasts from a patient with G_{M1}-gangliosidosis (type I) with fibroblasts of another variant with a combined β-galactosidase/neuraminidase deficiency (27). ß-galactosidase activity was assayed with 4-methylumbelliferyl-D-galactopyranoside as a substrate according to procedures described earlier (11). Both cell types were labelled with different fluorescent latex beads.

Two days after P.E.G. fusion, the mixed fusion population prior to flow sorting has a β-galactosidase activity of 69 nmol 4MU. mg protein^{-1}. hour^{-1}. The enzyme activity of the enriched heterokaryon fraction after flow sorting (109 nmol 4 MU. mg protein^{-1}. hour^{-1}) was also much higher than the parental cell populations (3.6 respectively 55 nmol 4MU. mg. protein^{-1}hour^{-1}).

This reappearance of enzyme activity in heterokaryons of enzyme deficient variant cell

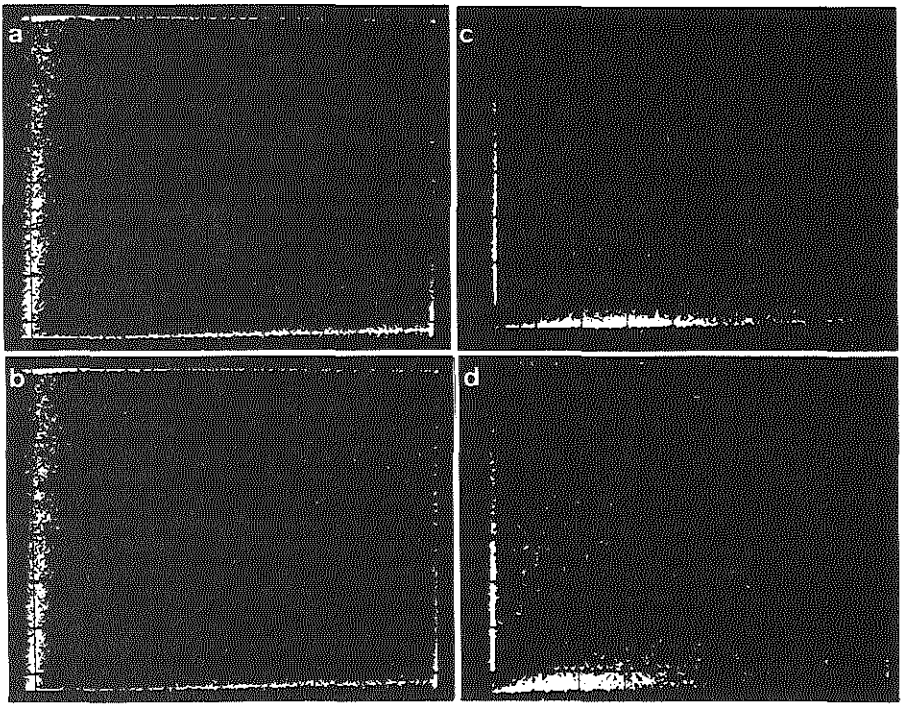

Fig. 3. Dot-plots of green- (x-axis) vs. red-fluorescence (y-axis) for two populations of fluorescent fibroblasts (red, green). 4000 cells.
a) latex-labelled cells after 24 hr of cocultivation.
b) latex-labelled cells after P.E.G.-induced fusion followed by 24 hr of cocultivation.
c) stearylamine-labelled cells after 7 h of cocultivation.
d) stearylamine-labelled cells after Sendai-induced fusion followed by 7 h of cocultivation.

strains is indicative of genetic heterogeneity. Since the enzyme activity of the flow-sorted heterokaryons showed a clear increase in comparison with the fusion population prior to sorting, this method of heterokaryon enrichment can be applied in cases where analysis on the mixed unsorted fusion populations does not give clear results.

To investigate the separate role of nucleus and cytoplasm in the mechanism of complementation, use has been made of cybrids: *i.e.* fusions of whole cells of one type with enucleated cells from another mutant (3). In general it is difficult to obtain pure anucleate cell fractions with all enucleation procedures, and even a small percentage of nucleated

Table 2. Distribution of viable nucleated cells after fusion and flow sorting.

Fluorescent labelling	Fusion procedure	% Gated[a]	n[b]	% Mono-karyons	% Bi-karyons	% Tri-karyons	% Tetra-karyons
Latex	P.E.G.	6	131	13.7	79.4	6.9	–
Latex	P.E.G.	3.5	495	14.5	76.8	8.1	0.6
Stearylamine	Sendai	7	581	6.3	84.3	6.7	2.6

a Percentage of cells within the deflection window of the FACS.
b Number of counted attached cells after sorting and culturing for 24 h.

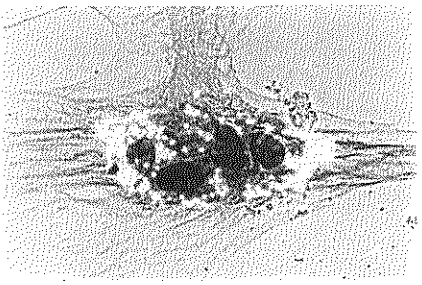

Fig. 4. Cybrid after Sendai-virus induced fusion of cytoplasts with beads (22, 23) and fibroblasts without beads (courtesy Dr. A. d'Azzo).

contaminants in the anucleate cell fraction makes a correct interpretation of the results rather difficult.

Therefore we devised methods for the isolation of an uncontaminated cytoplast fraction by two-colour flow sorting using latex beads as a green cytoplasmic, and Hoechst 33342 as a blue nuclear label (22, 23). After fusion of these fluorescent cytoplasts with other cell types, cybrids could be identified (Fig. 4). Further work is in progress to isolate these viable cybrids by flow sorting.

FLOW SORTING AFTER COCULTIVATION

The application of flow sorting in the study of lysosomal enzyme exchange was tested on populations of cells that were labelled with red- and green-fluorescent beads. Since the enzyme β-galactosidase is not exchanged between normal fibroblasts and ß-galactosidase deficient cells in confluent cultures (21), we used the same cell strains to test the purity of the sorted enzyme-deficient cells after a long period of cocultivation.

Normal cells (green) with a β-galactosidase activity of 725 nmol 4MU. mg protein^{-1}. hour^{-1} were cocultivated for 4 days with cells (red) from a patient with G_{MI}-gangliosidosis (βgal.act. 4.9 nmol 4MU. mg protein^{-1}. hour^{-1}). After trypsinization and flow sorting, the activity of the G_{MI} cells was 3.5 nmol 4MU. mg. protein^{-1}. hour^{-1}. This indicates that no contamination of normal cells was present in the sorted β-galactose deficient cell population after four days of cocultivation.

Two-colour flow sorting of labelled fibroblasts after a period of cocultivation seems therefore to be a promising technique to investigate intercellular interaction between cultured cells.

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PAPER 4

SELECTION OF PROLIFERATING CYBRID CELLS BY DUAL LASER FLOW SORTING

Isolation of Teratocarcinoma×Neuroblastoma and Teratocarcinoma×Endoderm Cybrids

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SUMMARY

An alternative method for the isolation of proliferating cybrid cells was developed, and was used to obtain teratocarcinoma × neuroblastoma and teratocarcinoma × endoderm cybrids. Enucleated neuroblastoma (or endoderm) cells labelled with fluorescent microspheres were fused with (HPRT-deficient) unlabelled teratocarcinoma cells. The cells in the fusion mixture were stained with the vital DNA stain Hoechst 33342 and the cybrids, containing both a fluorescent nucleus and fluorescent beads, were isolated by dual laser flow sorting. The purity of the sorted fraction, as determined by the percentage of cells showing HPRT activity, was 86 and 57% for the neuroblastoma and endoderm cybrids respectively. After single cell sorting in wells of Terasaki microtest plates, clones of proliferating cybrids were obtained with cloning efficiencies of 33% (neuroblastoma cybrids) and 10% (endoderm cybrids). The protein patterns of these clones and those of the parental cell lines were analysed by two-dimensional gel electrophoresis. A number of differences were found between the parental cell lines but all isolated colonies (sixteen neuroblastoma cybrids and eight endoderm cybrids) resembled the teratocarcinoma parent. These results therefore give no evidence for the existence of cytoplasmic factors in neuroblastoma or endoderm cells capable of inducing permanent differentiation of teratocarcinoma cells.

Cells can be fragmented into nuclear and cytoplasmic components with the use of cytochalasin B (CB) and centrifugation. These fragments, termed karyoplasts (minicells) and cytoplasts respectively, can be recombined with each other or with whole cells by cell fusion. A cybrid is the combination of a whole cell and a cytoplast (for review, see [1]).

Cybrid cells are used in the study of cellular differentiation and evidence has been obtained that at least some of the factors regulating the expression of differentiated functions may be localized in the cytoplasm. Thus, induction of hemoglobin synthesis by DMSO in Friend cells was permanently extinguished after fusion with neuroblastoma and fibroblast cytoplasts [2]. Permanent activation of the liver-specific enzyme phenylalanine hydroxylase was found after fusion of Friend cells with hepatoma cytoplasts [3]. Temporary activation of the liver-specific enzyme tyrosine aminotransferase was found in reconstituted cells formed from fibroblast karyoplasts and hepatoma cytoplasts [4]. Finally, temporary extinction of albumin production was observed in hepatoma cells after fusion with fibroblast cytoplasts [5].

Mouse teratocarcinoma cells are thought to correspond to cells of the early embryo and these cells are used as a model system

to study the early events of cellular differentiation (for review, see [6]). These cells retain the capacity to differentiate, and factors in the cytoplasm of differentiated cells might induce this differentiation. Cybridization experiments investigating this possibility have been reported by others [7–9].

Usually a chemical selection procedure is employed to isolate cybrid cells, most often utilizing cytoplasmic donor cells which carry the cytoplasmically inherited trait of chloramphenicol (CAP) resistance [10, 11]. However, cybrids have also been isolated after iodoacetate poisoning [12] and by making use of temporary HAT resistance [8].

In earlier studies we were able to isolate fibroblast cybrids as a very pure population by flow sorting, using two different cytoplasmic fluorescent markers [13]. In this report we show that cybrids can be obtained by flow sorting using a slightly different approach, also applicable when the acceptor cell is unable to take up cytoplasmic label. For this purpose we made use of dual laser flow sorting, utilizing cytoplasts labelled with fluorescent beads and the vital DNA stain Hoechst 33342. Teratocarcinoma cells were fused with neuroblastoma- and endoderm cytoplasts and after Hoechst staining the heterofluorescent cells were selected and cloned by single-cell sorting. Cybrid colonies arising from these cells were isolated and their two-dimensional electrophoretic protein patterns were compared with those of the parental cells, to investigate whether cytoplasmic factors from the differentiated cells had influenced the undifferentiated teratocarcinoma cells.

MATERIALS AND METHODS

Cell culture, enucleation and fusion

The cell lines used were mouse teratocarcinoma PCC_1AZA_1 [14], mouse neuroblastoma NB41A3 (American Tissue Type Collection, CC1147) and PSA5EA4. PSA5E cells are endoderm-like cells isolated from the pluripotent teratocarcinoma cell line PSA5 [15] and were a gift from Dr M. J. Evans (Cambridge University, UK). PSA5EA4 is a tetraploid subclone of this cell line isolated in our laboratory. All cells were grown in Ham's F10 or Dulbeccos modified Eagle Medium containing 10% FCS, penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells to be enucleated were grown for one day in the presence of 1.83 μ green fluorescent beads ($\pm 1.1\times10^8$ beads/ml; Polysciences cat. no. 9847). Enucleation and purification of cytoplasts was done as described before [16, 17] using Percoll (Pharmacia) gradients and scatter and Hoechst fluorescence as the sorting parameters. Teratocarcinoma cells and sorted cytoplasts were fused in suspension (5×10^5–10^6 of each in a fusion volume of 0.5 ml) using inactivated Sendai virus (125 HAU) according to [18].

Cybrid isolation

Fused cells which had been cultured overnight were trypsinized and stained with Hoechst 33342 (10 μM, 45 min at 37°C). Flow analysis and sorting was performed with a FACS II cell sorter (Becton Dickinson) equipped with a commercially available dual laser excitation system. Excitation was at 351–364 nm (40 mW constant output) and at 488 nm (100 mW constant output) using Spectra Physics argon ion lasers (model no. 164-05 and 164-06). A KV 399 filter was used to block scattered UV light. In front of the 'blue' photomultiplier (PM) K420 and SP440 filters were placed, and in front of the "green" PM K515 and K520 filters were inserted. (All filters were from Schott except SP440, which was from Ditric Optics.) The sorter was calibrated with the fluorescent beads used for cell labelling and with Hoechst 33342 stained teratocarcinoma cells. Cells were directly cloned [19, 20] by single-cell sorting in separate wells of a Terasaki microtest plate containing 20 μl of growth medium (F10) and allowed to settle for 1 h. All wells were then microscopically examined and wells containing only one cell with eight or more beads were noted. The next day the dishes were totally filled with growth medium. The wells were inspected a second time for the presence of dividing cells containing beads after 3 days of culture. Colonies arising in these wells were picked, expanded to $5–10\times10^6$ cells and stored in liquid nitrogen.

Measurement of HPRT activity

Cells were sorted onto coverslips, incubated with [^{3}H]hypoxanthine (10 μCi/ml sp. act. 1 Ci/mmol) for 10 h and fixed. With a fluorescence microscope the position of separately lying cells containing eight or more beads was noted. The slides were then processed for autoradiography (Ilford K2 emulsion) and the same cells were scored for the presence of silver grains.

Two-dimensional gel electrophoresis

For cell labelling 200 000 cells were seeded in 3 cm dishes in normal growth medium (F10). After 8 h the

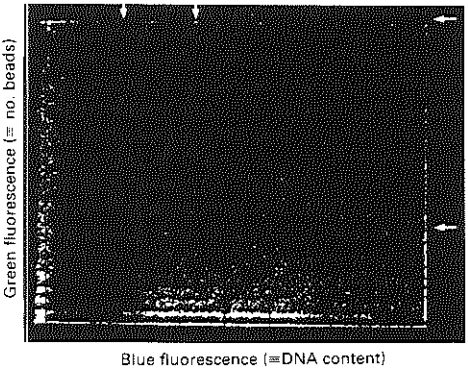

Fig. 1. Flow analysis using dual laser excitation of a fusion mixture (endoderm cytoplasts containing green fluorescent beads fused with unlabelled teratocarcinoma cells) after staining with Hoechst 33342. 10000 cells were analysed. Arrows indicate sorting window.

medium was replaced with 1.5 ml fresh medium containing 20 μCi/ml [^{35}S]methionine (Radiochemical Centre, Amersham; sp. act. >600 Ci/mmol) and cells were labelled for 20 h. Two-dimensional electrophoresis was performed essentially according to O'Farrell [21]. Triton X-100 was used instead of NP-40 and ampholines (LKB) were in the range 5–8 (1.6% w/v) and 3.5–10 (0.4% w/v). Samples for the first dimension gels contained 5×10^5 acid-precipitable cpm. First-dimension gels were 11.5 cm long; second-dimension gels were linear 7–18% gradients (10.5 cm long). Gels were fixed, impregnated with PPO and dried onto Whatman 3 MM paper. Fluorography was performed using preflashed [22] Kodak X-omat R films (exposure time 3 days). For determination of the pH gradient in the first dimension, parallel gels were measured with a contact pH electrode. Calibration in the second dimension was done with radioactive MW markers (NEN).

RESULTS

Isolation of cybrid cells by flow sorting

Cytoplasts labelled with fluorescent microspheres were prepared from endoderm and neuroblastoma cells. The cytoplasts were then fused with the teratocarcinoma cells and the fusion mixture was cultured overnight to recover. The next day the cells were trypsinized, stained with Hoechst 33342 and sorted. A typical example of a dot plot is shown in fig. 1. The cells emit discrete quantities of green fluorescence (Y-axis), corresponding to the number of beads they contain. For example cells containing zero, one and two beads can be seen as distinct horizontal lines. On the blue fluorescence axis (X-axis) cells are distributed according to their DNA content and cells with G1 and G2 DNA content corresponding to G1 (2c) and G2 (4c) of PCC4 can be discerned. Unfused cytoplasts or cytoplasts fused with themselves (cells with beads but without a fluorescent nucleus) can be seen on the Y-axis and unfused teratocarcinoma cells or fusion products of these cells (nucleated cells without beads) can be seen on the X-axis. In between these populations cybrid cells are found (nucleated cells also containing fluorescent beads). Cells containing eight or more beads with 2c DNA content were selected (sorting window shown in fig. 1, about 2% of the total population) and cloned in Terasaki microtest plates. 20% of the wells actually contained a single cell with eight or more beads after sorting, the remainder being mostly empty wells. A few wells contained an aggregate of a cell and a cytoplast. In the case of the endoderm cybrids 6–14% (two experiments) of the sorted cybrid cells proliferated and formed a colony. For the neuroblastoma cybrids the cloning efficiency was 33%. Cloning efficiencies of

Table 1. *Cloning efficiences of cybrid- and unfused cells*

Cytoplasmic donor	Cloning efficiency (%)[a]	
	Cybrids	Unfused cells
Endoderm	6	55
	14	43
Neuroblastoma	33	85

[a] Percentage outgrowth of sorted single cells.

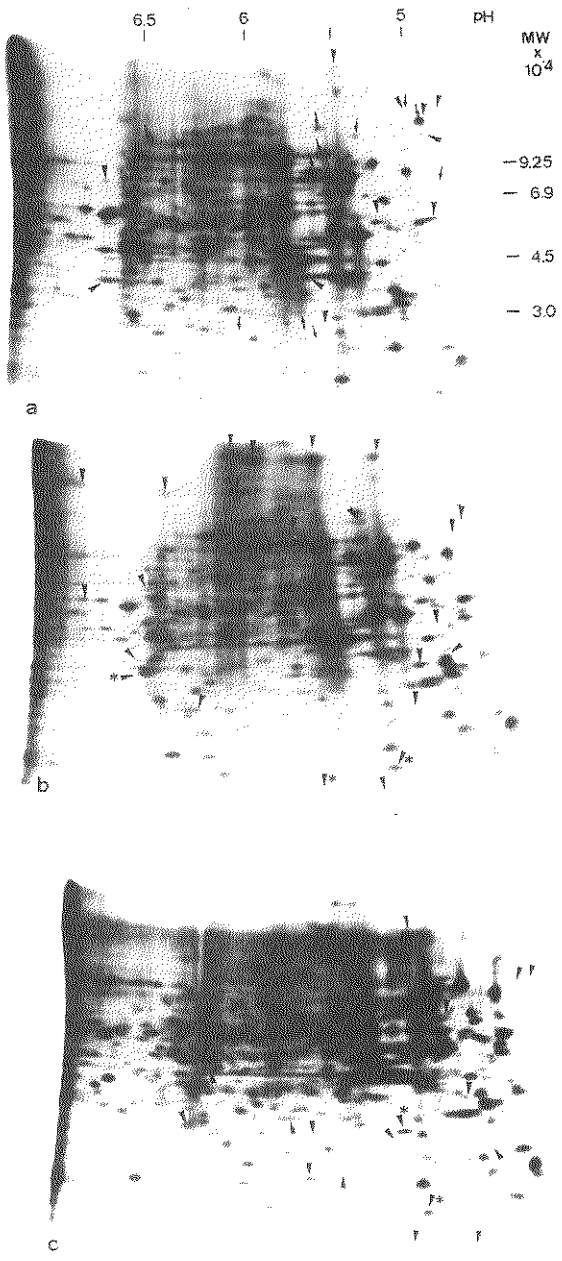

Fig. 2. Two-dimensional protein patterns of parental cell lines. (*a*) Teratocarcinoma (PCC4AZA1); arrowheads indicate spots not present or present in reduced amount in endoderm cells; arrows, differences with neuroblastoma cells. (*b*) Endoderm (PSA5EA4). (*c*) neuroblastoma (CC1147); arrowheads indicate spots not present or present in reduced amount in PCC4AZA1. Some of the more prominent spots have also been labelled with an asterisk.

unfused teratocarcinoma cells (cells in G1 but without beads sorted from the same fusion mixture) varied, but were always higher than those of the cybrid cells (table 1). Eight endoderm cybrids and sixteen neuroblastoma cybrids were isolated.

Validity of cybrid isolation

Since PCC4AZA1 cells are HPRT$^-$, cybrids should show a transient HPRT activity brought about by enzyme present in the cytoplast [8, 23]. This activity was therefore used to determine the efficiency of the isolation procedure. In each experiment a sorted cybrid fraction was cultured in the presence of [^{3}H]hypoxanthine. The percentage of cells containing eight or more beads which had incorporated [^{3}H]hypoxanthine was 86% for the neuroblastoma cybrids (number of cells counted (n) 44). For the endoderm cybrids the percentage was about 57% (two experiments: 47% (n, 30) and 67% (n, 63)).

Analysis of isolated cybrid clones

Morphologically all isolated clones resembled the teratocarcinoma cells. For a more detailed biochemical analysis the two-dimensional electrophoretic patterns of the proteins of the clones were compared with those of the parental cell lines. Fig. 2 shows the patterns of the parental cells. About 500 different spots were resolved and several differences, shown in fig. 2, could be seen between the teratocarcinoma cells and the differentiated cells. In CC1147 twelve spots were found which were not visible in PCC4AZA1 and five spots were significantly more dense. Likewise in PCC4AZA1 seven spots were found not visible in CC1147 and five spots were more dense. Similarly PSA5EA4 had fourteen spots not found in PCC4AZA1 and ten spots which were more dense, and PCC4AZA1 had nine spots not found in PSA5EA4 and three spots which were more dense. The patterns of all isolated cybrid colonies resembled that of the teratocarcinoma cells and none of the spots specific for the cytoplasmic donor cells had appeared. Therefore, at this resolution all isolated colonies resembled the teratocarcinoma parent cells.

DISCUSSION

We have previously shown that it is possible to isolate fibroblast cybrids from a fusion mixture by flow sorting using fluorescent microbeads [13]. A different approach was adopted in this study since the teratocarcinoma cells we have used take up fluorescent microspheres very poorly. In this procedure, the fusion products of unlabelled cells and cytoplasts labelled with green fluorescent beads were selected after DNA staining with Hoechst. Since the fluorescent beads and the Hoechst dye are excited at different wavelengths, use of dual laser excitation (principle described in [24]) was necessary. Only those heterofluorescent cells with a DNA content corresponding to G1 of PCC4 and containing eight or more beads were selected. By sorting cells with this DNA content any not enucleated parental cytoplasmic donor cells which could be present in a small amount (cytoplasts preparations are more than 99% pure) will be eliminated, since these cells are pseudotetraploid (CCL147) or tetraploid (PSA5EA4). Furthermore, fusion products containing more than one teratocarcinoma nucleus will not be sorted. The sorting criterium of eight beads was chosen to exclude cells with non-specifically bound beads. The entire procedure including the microscopical checks after sorting ensured that true single cell colonies originating from

cells containing eight or more beads were isolated.

The validity of the cybrid isolation was tested by measuring [^{3}H]hypoxanthine incorporation in the sorted cybrids. This analysis showed that whereas 86% of the neuroblastoma cybrids was HPRT$^+$, only about 57% of the sorted endoderm cybrids had this activity. We consider only those cells as 'true' cybrids in which a transfer of cytoplasmic protein could be demonstrated by the presence of HPRT activity after the entire procedure. Assuming equal outgrowth of HPRT$^-$ and HPRT$^+$ cells the data show that fourteen of the sixteen isolated neuroblastoma cybrids colonies and five of the eight endoderm colonies should be 'true' cybrid colonies.

Several methods have been described for the isolation of cybrids. The most commonly used method of CAP selection results in efficient killing of unfused cells (see, e.g. [10, 11]). Therefore our method which can yield a panel of colonies, a number of which will be 'true' cybrids, appears to have a slightly lower selectivity. However, our method may be especially useful in cases where no CAP-resistant mutants can be isolated (e.g., primary cells) or when interspecific crosses are investigated, since it has been reported that interspecific cybrids can be isolated less easily or not at all using CAP selection [25].

The protein patterns of the isolated colonies were analysed by two-dimensional electrophoresis. With this technique changes in protein patterns upon differentiation of teratocarcinoma cells have been demonstrated [15, 26]. The 2D-patterns we have obtained show differences between the teratocarcinoma cells and the differentiated endoderm and neuroblastoma cells. At least some of these differences should reflect changes in protein pattern due to the different differentiated states of the cells, especially in the case of the endoderm cells which have the same genetic background as the teratocarcinoma cells. Therefore our finding that all isolated colonies resemble the teratocarcinoma cells with respect to their 2D-pattern shows that at the resolution obtained these colonies consist of undifferentiated teratocarcinoma-like cells.

Even though a large number of colonies were analysed, our results thus give no evidence for the existence of cytoplasmic factors in neuroblastoma (or endoderm) cells capable of inducing permanent differentiation of teratocarcinoma cells. We have assumed equal outgrowth of HPRT$^-$ and HPRT$^+$ cybrid cells (see above). The possibility has to be considered, however, that induction of differentiation is accompanied by a blocked growth of the induced cells (HPRT$^+$). Thus it might be possible that clones originating from uninduced (HPRT$^-$) cells would preferentially be isolated. In this context the reduced plating efficiency of the cybrids as compared with the unfused cells (table 1) has to be considered. In the case of the neuroblastoma cybrids (86% HPRT$^+$, plating efficiency 33%) at least about half of the clones should have originated from HPRT$^+$ cells. But in the case of the endoderm cybrids ($\pm$57% HPRT$^+$; plating efficiency $\pm$10%) HPRT$^-$ cells preferentially growing out might account for all isolated clones. However, we do not think that the reduction of the plating efficiency of the cybrids should be interpreted as evidence for reduced growth of induced (HPRT$^+$) cells. It also occurs in the case of the neuroblastoma cybrids, but there it is clear that not all isolated clones are formed from HPRT$^-$ cells. One explanation might be that the reduced outgrowth of the cells is caused by the intracellular presence of the

beads. In conclusion we do not think that in the case of the endoderm cybrids our results are likely to be explained by selective outgrowth of HPRT$^-$ cells. However, we cannot exclude this possibility.

Altered differentiated phenotypes upon cybridisation have been reported [2–5]. In other cases cybrids resembled the nuclear donor parent (see, e.g. [28], and references therein). Teratocarcinoma cybrids have also been produced by fusion of these cells with melanoma [27], Friend cell [7], fibroblast [7] and myoblast cytoplasts [8, 9]. No permanent alteration of the undifferentiated teratocarcinoma parent was observed. The teratocarcinoma cells used in our study can differentiate into primitive neuronal tissue in vivo and into endoderm in vitro. Our observations extend the results mentioned above and show that the teratocarcinoma cells, even when capable of differentiating in the direction of the cytoplasmic donor cell, are not triggered to differentiate by cytoplasmic factors from these cells, not even in the case of the most closely related endoderm cells.

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PAPER 5

Flow Sorting in the Study of Cell-Cell Interaction[1]

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Undifferentiated mouse teratocarcinoma cells were cocultivated with differentiated mouse endoderm cells in order to study the possible induction of teratocarcinoma cell differentiation.

A difference in DNA content between the two cell types was experimentally introduced to enable the reisolation of the teratocarcinoma cells after cocultivation. Pseudotetraploid (2s) endoderm cell lines were produced from pseudodiploid (1s) cells by treatment of these cells with cytochalasin B and flow sorting of tetraploid cells, using Hoechst 33342 as a viable DNA stain, with subsequent cloning of sorted single cells.

In model experiments, where mixtures of 1s teratocarcinoma and 2s endoderm cells were stained with Hoechst 33342, the teratocarcinoma cells could be reisolated with a purity of about 97%.

After a cocultivation period of 24 days viable teratocarcinoma cells could be isolated from the cocultivation mixture with a purity of 95%. Two dimensional analysis of the protein pattern of these cells indicated that cocultivation did not induce a differentiated (endoderm) pattern.

Therefore according to this analysis the teratocarcinoma cells were not induced to differentiate during a 24 day cocultivation period. The method described offers excellent possibilities for studying cell-cell interaction *in vitro*.

Key terms: Cell-cell interaction, teratocarcinoma, flow cytometry, 33342 Hoechst, tetraploidization, cocultivation, differentiation

For the study of the early events of cellular differentiation mouse embryonal carcinoma (EC) cells are used as a model system (3, 11). Several lines of evidence suggest that the *in vitro* and *in vivo* environment of the EC cells may influence their behavior. Thus, aggregation of cells may result in induction of differentiation (11) and furthermore injection of one EC cell into a blastocyst will cause this EC cell to differentiate normally and to contribute to the embryo (11). An environmental change *in vitro* may also be created by cocultivation of EC cells with differentiated cells. Since endoderm is the first cell type that will originate from EC cells upon differentiation, we were interested in these differentiated endoderm cells as a possible inducing cell type.

After cocultivation of EC and endoderm cells, one should be able to reisolate the EC cells in order to analyze the possible induction of differentiation. Therefore, endoderm cells were first made tetraploid by cytochalasin B (cyt B) treatment (2) and tetraploid cell clones were subsequently

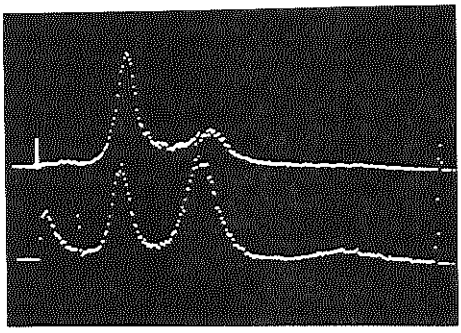

FIG. 1. DNA fluorescence distribution of endoderm cells (PSA5E) stained with Hoechst 33342 after 20 hr of culturing in the presence of 5 μg/ml cytochalasin B (*lower*) compared with that of untreated cells (*upper*). *X-axis*: relative fluorescence intensity; *Y-axis*: number of cells. Note the increase in cells with a DNA content corresponding to G2 of PSA5E or more after cytochalasin B treatment.

[1] This work was supported by Grant 92-48 from The Netherlands Organization for the Advancement of Pure Research.

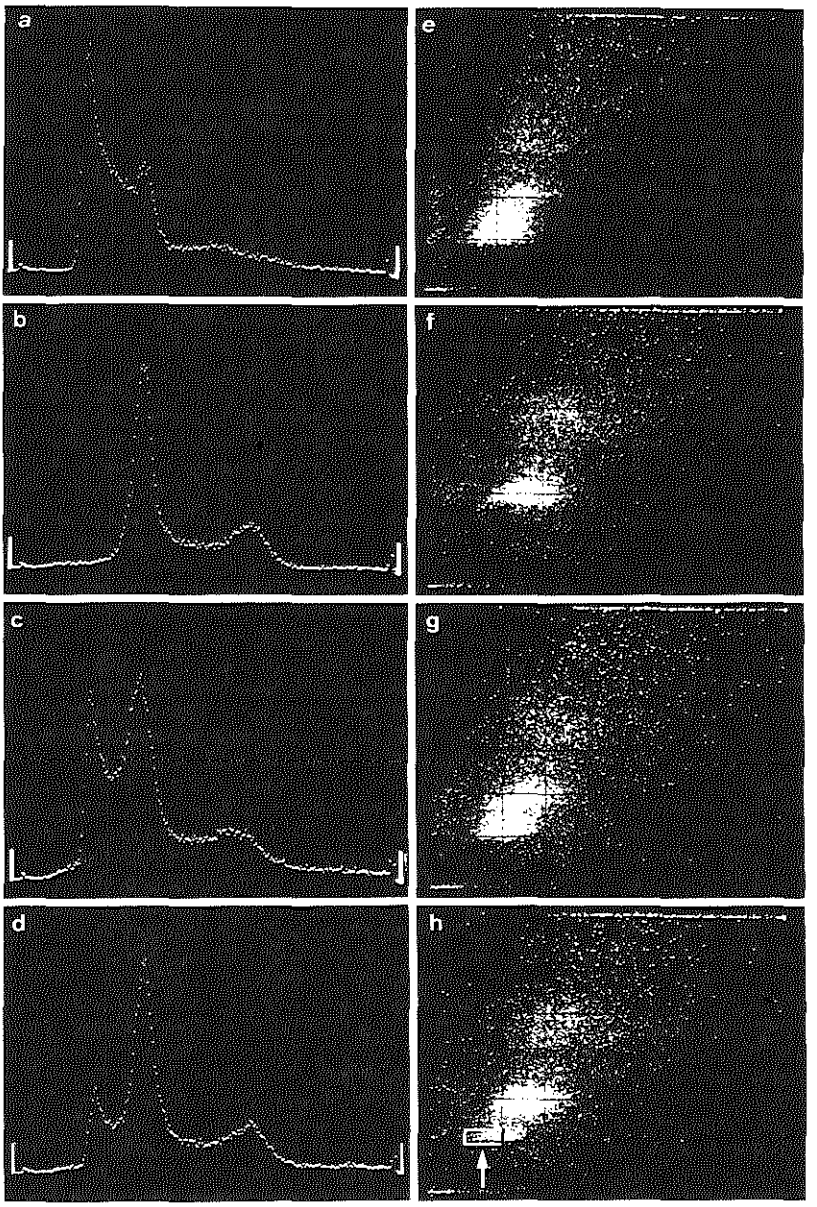

FIG. 2. DNA fluorescence distributions (*a–d*) and dot plots (*e–h*); *a* and *e*: teratocarcinoma cells (PCC4AZA1: 1s); *b* and *f*: endoderm cells (PSA5EA4: 2s); *c* and *g*: 1:1 mixture of teratocarcinoma (1s) and endoderm cells (2s); *d* and *h*: 1:2 mixture of teratocarcinoma (1s) and endoderm cells (2s). Cells were stained with Hoechst 33342. *a–d*: *X-axis* relative fluorescence intensity; *Y-axis* number of cells. *e–h*: *X-axis* scatter; *Y-axis* relative fluorescence intensity (4000 cells). The position of the sorting window is indicated in *h*. Note also the presence of some cells with fluorescence lower than G1 of PCC4AZA1 in endoderm (2s) cells.

produced by single cell sorting (14,17). After cocultivation of teratocarcinoma cells (pseudodiploid, 1s) and endoderm cells (pseudotetraploid, 2s), the DNA content of the cells was used as an internal marker to isolate the EC cells by flow sorting using Hoechst 33342 as a vital fluorescent DNA stain (1). The protein pattern of these sorted cells was compared with that of independently cultivated EC cells and endoderm cells to investigate whether induction of a differentiated (endoderm) protein pattern had taken place in the cocultivated EC cells.

Materials and Methods

Cell culture: EC cells PCC4AZA1 without hypoxanthine phosphoribosyl transferase activity ($HPRT^-$) (6) and endoderm cells PSA5E (10) and PSA5EA4 (tetraploid) were routinely grown in Ham's F10 medium supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml) and streptomycin (0.1 mg/ml). For cocultivation, cells were grown in Dulbecco's modified Eagle's medium (DME) with the same additions. PCC4 and PSA5EA4 cells have different growth rates and were passaged at 1:12 and 1:6 ratios, respectively, every 2 days. Therefore, in order to prevent overgrowth of PCC4AZA1 in the cocultivation mixture, cocultivation was started by mixing 10^6 PSA5EA4 cells and 25,000 PCC4AZA1 cells in a T-flask (40 cm^2). This mixture was subcultured every 2 days at 1:12 with an extra addition of 500,000 PSA5EA4 cells. With this procedure the relative amount of PCC4 was approximately 30% after 22 days of cocultivation. In order to increase the percentage to about 50%, two days before sorting the cocultivation mixture was passaged at 1:6 without addition of PSA5EA4.

For harvesting of cells a stock solution of 0.5 g/liter trypsin and 0.2 g/liter EDTA in PBS was prepared. PCC4AZA1 and PSA5EA4 cells were treated routinely with a 1:5 and 1:2, respectively, dilution of the stock solution. To prevent detachment in sheets of the cocultivated cells, the cells were first trypsinized mildly with a 1:10 dilution of the stock and then the same volume of undiluted stock was added to detach still attached cells.

Cell tetraploidization: Duplication of DNA content was accomplished by cytochalasin B treatment (2,4). PSA5E cells growing exponentially were cultured for 20 hr with 5 μg cytochalasin B (Serva) per ml of medium. After this period the medium was replaced by fresh medium without cytochalasin. One hour after recovery the cells were harvested for flow sorting.

Flow analysis and sorting: Vital staining of DNA was done by incubating cells in medium containing 10 μM Hoechst 33342 (1,8) for 45–60 min at 37°C. Nonvital DNA staining with ethidium bromide was performed according to Vindelov (18 (see also 13)). The cells were analyzed and sorted using a FACS II cell sorter (Becton & Dickinson, Sunnyvale, CA) either equipped with a 5 W argon ion laser (Spectra Physics, 164-05, Mountainview, CA) and barrier filters KV400 and K445 (Schott Optical Glass Inc., Duryea, PA) for measurement of Hoechst fluorescence (exc. 351,1 and 363,8 nm: 40 mW) or with a 2 W argon ion laser (164-01) and barrier filter K580 (Schott) for EB fluorescence (exc. 488 nm, 100 mW). The Hoechst stained cells were sorted directly into plastic Petri dishes containing growth medium for subsequent culturing. When redistribution of the cells was required, the cells were sorted on agar coated Petri dishes, washed and divided over more than one dish. Single cell cloning in 96 multiwell plates was done as described elsewhere (16).

Autoradiography of cells and 2D-electrophoresis: For autoradiographic detection of hypoxanthine phosphoribosyl transferase (HPRT) activity, cells were seeded on glass coverslips and incubated in medium containing [^{3}H]hypoxanthine (spec. act 1 Ci/mmol; Radiochemical Centre, Amersham). For the mixing experiment cells were incubated for 6 hr at 10 μCi/ml, while after cocultivation a labeling period of 20 hr (3 μCi/ml) was used. The cells were fixed with Bouin's fixative and the slides were processed for autoradiography (Ilford K2 emulsion, Ilford Ltd., Basildon, England).

For two-dimensional electrophoresis sorted cells were incubated with [^{35}S]methionine in F10 growth medium (~ 25 μCi/ml; spec.act. > 600 Ci/mmol, Radiochemical Centre, Amersham, England) for 20 hr. Two dimensional electrophoresis was done according to O'Farrell (12) with slight modifications as described elesewhere (16).

Results

Production of tetraploid endoderm clones: Endoderm cells were cultured in the presence of cyt B for 20 hr. Microscopical examination at this time showed that about 50% of the cells were binuclear. Flow analysis of Hoechst 33342 stained cells showed a large increase in number of cells with a DNA content corresponding to G2 of PSA5E or more (Fig. 1). These cells were sorted and allowed to grow for several days. Subsequently, cells were again stained with Hoechst 33342 and single cells with a DNA content corresponding to G2 of 2s cells were directly cloned by single cell sorting in wells of 96-multi-well plates (cloning efficiency 10%).

Flow analysis using EB-staining on expanded clones showed that 6 out of 11 colonies consisted of 2s cells. One of these clones (PSA5EA4) was selected for cocultivation experiments. This cell line was stable; no reduction of DNA content was found after prolonged subcultivation.

Flow sorting of mixed cells: PCC4 and PSA5EA4 cells were mixed in 1:1 and 1:2 ratios and analyzed after staining with Hoechst 33342. The DNA distributions and dot plots of DNA fluorescence *versus* scatter of PCC4, PSA5EA4 and 1:1 and 1:2 mixtures is shown in Figure 2. Cells were sorted from the mixtures as indicated in Figure 2*h*, sorting cells with a DNA content equal to G1 of PCC4AZA1. To determine the purity of the sorted fraction, the cells were incubated with [^{3}H]hypoxanthine for 6 hr and then the viable attached cells were fixed and autoradiography was performed. Since PCC4AZA1 cells lack the enzyme HPRT, these cells are unable to incorporate the label and will show no grains upon autoradiography. In contrast the $HPRT^+$ PSA5EA4 cells are heavily labeled. As determined by this method the sorted cells contained 97.3% (n = 2666) and 96.8% (n = 2256) $HPRT^-$ cells starting with the 1:1 and 1:2 mixture, respectively.

Separation and analysis of cocultivated cells: PCC4AZA1 and PSA5EA4 cells were cocultivated at high density (25,000–150,000 cells/cm^2) for a period of 24 days. After this period the cells were stained with Hoechst 33342

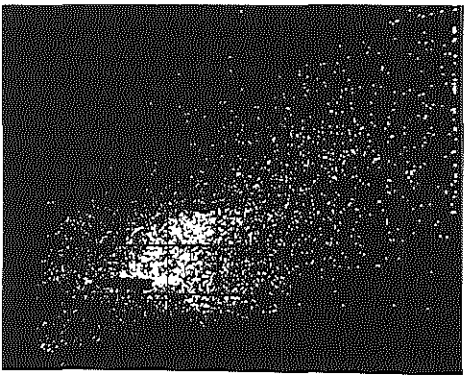

FIG. 3. Dot plot of a population of cocultivated cells (24 days). *X-axis*: scatter signal, *Y-axis*: relative fluorescence intensity (4000 cells). Black rectangular shows sorting window for (1s) PCC4AZA1 cells (in G1).

and the 1s PCC4 cells in G1 were sorted. A dot plot showing scatter *versus* DNA distribution is shown in Figure 3. The sorted area is indicated by the black retangular area.

About 200,000 cells were sorted from the cocultivation mixture and 20,000 cells were incubated with [^{3}H]hypoxanthine for determination of HPRT activity in the sorted cells. The percentage EC (HPRT$^-$) cells as determined by autoradiography was 95% (n=3428). The remainder of the sorted

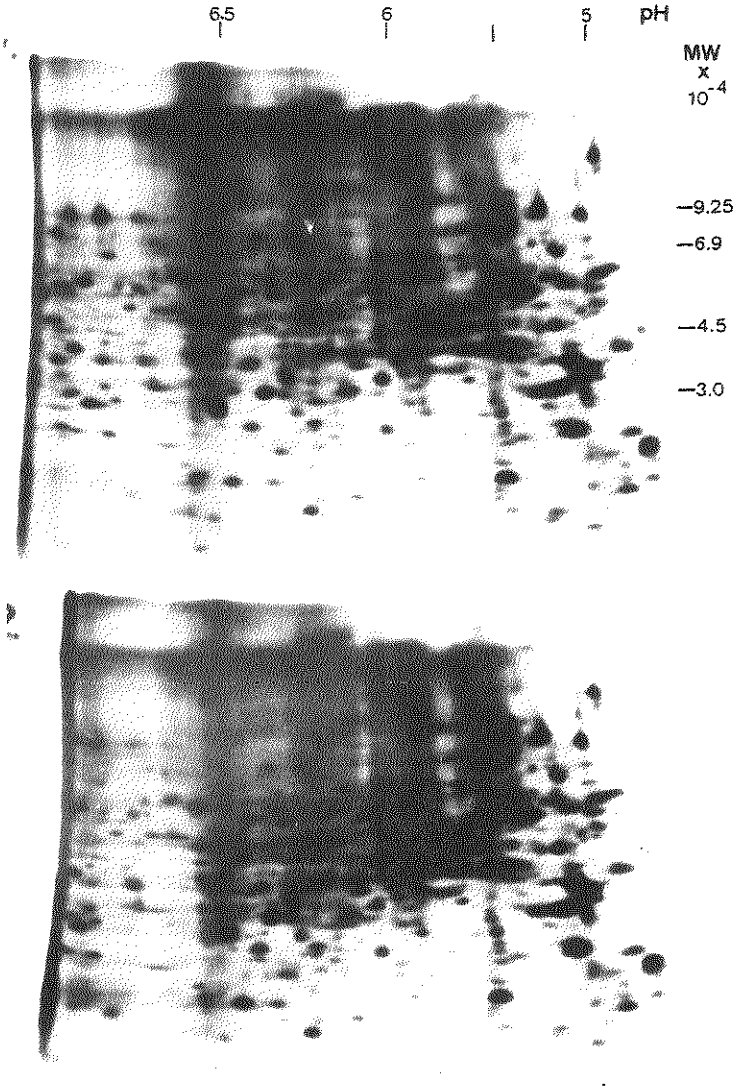

FIG. 4. Two dimensional gel analysis of [^{35}S]methionine labeled proteins from about 60,000 sorted cells. *Upper*) PCC4AZA1 cultured separately and sorted in G1; *lower*) PCC4AZA1 from the cocultivation mixture sorted as indicated in Figure 3.

cells was incubated with [^{35}S]methionine for protein labeling. As a control separately cultured PCC4AZA1 and PSA5EA4 cells were also stained, sorted in G1 and radioactively labeled.

The two dimensional protein pattern of the teratocarcinoma cells sorted from the cocultivation mixture was identical with that of the non-cocultivated teratocarcinoma cells (Fig. 4). Furthermore the morphology of the sorted EC cells (HPRT$^-$) resembled the parent teratocarcinoma cells rather than the endoderm cells.

Discussion

The aim of this study was to investigate whether teratocarcinoma cells could be induced to differentiate by cocultivation with differentiated (endoderm) cells. The reason for using endoderm cells as the differentiated cell is that these cells are the first differentiated cell type to appear upon differentiation of teratocarcinoma cells (11). Furthermore we have observed (results not shown) that the endoderm cells show extensive metabolic cooperation (5, 9) with the PCC4AZA1 cells. Therefore apart from molecules diffusing through the medium, small molecules also may pass from the endoderm cells to the EC cells through gap junctions.

To be able to see a possible induction of differentiation in the teratocarcinoma cells, it was necessary to reisolate these cells from a cocultivation mixture. A difference in DNA content between the two cell types was artificially introduced by duplicating the DNA content of the 1s endoderm cells. Thus, the DNA content was used as an internal marker for the two cell types making possible a separation after a long period of cocultivation.

Our results first show that by making use of the vital DNA stain Hoechst 33342 and a two step isolation procedure using the cell sorter, proliferating clones consisting of 2s cells could be isolated with great efficiency (~50%), starting from the mixture of cells after cyt B treatment. Since trypsinized endoderm cells never form an ideal cell suspension the contaminating 1s clones probably arose from doublets of sorted 1s cells which were attached to each other.

Mixing experiments were performed to test out the sorting procedure. These results showed that a high degree of purity (about 97%) could be obtained while sorting 1s cells in G1. The purity of the sorted cell fraction depended very much on proper window setting. As can be seen with Hoechst stained PSA5EA4 cells (Fig. 2 *b* and *f*), some cells occurred with an apparent DNA content lower than G1 of PSA5EA4 and even lower than G1 of PCC4. These cells were not present when the PSA5EA4 cells were analyzed using EB as a nonvital DNA stain (results not shown). Therefore, the PSA5EA4 cell culture did not contain cells with subtetraploid DNA content but in the population cells were present with a decreased stainability with Hoechst 33342. Such differential staining for lymphocytes by Hoechst 33342 has also been reported by others (7). Since the endoderm cells had a larger scatter signal (Fig. 2) the purity of the sorted cell fraction was enhanced to 97% by selecting only cells in a small sorting window (Fig. 2*h*).

Since the cells that were used do not show contact inhibition, cells were subcultured every 2 days during the long cocultivation period of 24 days in order to prevent overcrowding. Under these conditions a very high density was reached at the end of the 2-day period. Since the PCC4 cells also tend to form processes touching PSA5EA4 cells, density after subculturing was high enough for metabolic cooperation to occur at all times during cocultivation.

From a mixture of cocultivated endoderm and teratocarcinoma cells, the flow sorted cells contained 95% PCC4AZA1 cells. Since these cells were vitally sorted, they could incorporate [^{35}S]methionine and a two-dimensional electrophoretic analysis of synthesized protein just after cocultivation and sorting was possible. Since differences exist between the protein patterns of teratocarcinoma and endoderm cells (16), an induction of differentiation after cocultivation should show up in the 2D gel pattern. The protein pattern of the cells sorted from the cocultivation mixture is nearly identical with that of non-cocultivated PCC4AZA1 cells. Therefore we conclude that with the present method of protein analysis no induction of differentiation could be seen to occur in the majority of the PCC4 cells after a 24-day cocultivation period.

In this context it must be mentioned that a small amount of differentiated cells would not be detected by this method since the contribution of 5% PSA5EA4 cells did not show up in the 2D pattern. However our conclusion that no induction takes place is strengthened by the fact that all sorted HPRT$^-$ cells after cocultivation morphologically resemble EC cells.

The PCC4AZA1 cells used in this study, although capable of undergoing limited differentiation both *in vivo* and *in vitro*, virtually will not differentiate under normal tissue culture conditions. Apparently this restriction cannot be overcome by factors from the endoderm cells diffusing through the medium or by low molecular weight substances passing through gap junctions. This is in line with the observations of Rosenstraus and Spadoro (15) that in a mixed culture of nullipotent and pluripotent EC cells the nullipotent cells were not induced to differentiate as were the pluripotent EC cells.

Acknowledgments

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PAPER 6

Fluorescence Polarization of Six Membrane Probes in Embryonal Carcinoma Cells After Differentiation as Measured on a FACS II Cell Sorter

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Fluorescence polarization measurements on a FACS II cell sorter were compared with static measurements on a spectrofluorimeter using calibration solutions and Hoechst 33258-labeled cells. For the flow cytometric measurements on the FACS we used a pseudodepolarizer for normalization of the output of the two photomultipliers. The results showed that fluorescein and fluoresceinated bovine serum albumin (BSA) solutions gave identical values on both instruments. The mean value for fluorescence polarization of Hoechst 33258-labeled cells as measured on the FACS was the same as the value obtained with the spectrofluorimeter.

Subsequently the fluorescence polarization of six different membrane probes was determined using differentiating embryonal carcinoma cells as a model system. Differentiation was induced by treatment of the cells with retinoic acid together with cyclic AMP. With diphenylhexatriene (DPH) the fluorescence polarization increased from $I_{\parallel}/I_{\perp}$ = 1.55 to 1.74 upon differentiation. With a charged analog of DPH (TMA-DPH) fluorescence polarization increased from $I_{\parallel}/I_{\perp}$ = 1.87 to 2.02. No appreciable changes in fluorescence polarization were observed in this cell system when anthroyloxysterate probes (12-AS, 9-AS, 6-AS, 2-AS) were used.

Key terms: Fluorescence polarization, fluorescent membrane probes, embryonal carcinoma, differentiation

Measurement of fluorescence polarization (FP) of membrane-embedded fluorecent probes may yield information about the "fluidity" of biological membranes (for review, see 15, 16). With flow cytofluorometry it is possible to perform FP measurements on a single-cell basis and so reveal possible heterogeneity in the cell populations analyzed. An extensive review on FP measurements in flow systems has recently appeared (8). Several flow systems with different optical configurations have been used (1,5,8,10,13) and a number of optical and electronic factors that may complicate polarization measurements in flow have been enumerated (8).

The membrane probe most widely used for polarization measurements is 1,6-diphenyl-1,3,5-hexatriene (DPH). This probe also gives intracellular labeling when intact cells are used (2,3). Recently a charged analog of DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH), has been synthesized (4,12), which might show less internal labeling. A group of anthroyloxy-fatty acid probes with the anthroyloxy group attached to the alkyl chain at different positions has also been described (18). These probes offer the possibility of measuring "fluidity" at different depths in the bilayer (19).

Mouse teratocarcinoma cells offer an experimental system to evaluate the behavior of fluorescent membrane probes in intact cells. These undifferentiated cells (for review see 11) have the capacity to differentiate upon treatment with chemical inducers. With the technique of fluorescence photobleaching recovery it has been demonstrated that the lateral diffusion of a fluorescent probe in the plasma membrane of F9 teratocarcinoma cells is reduced after retinoic acid-induced differentiation (14). Moreover, it was demonstrated that the "apparent" membrane microviscosity was enhanced during differentiation of another teratocarcinoma cell strain (OC15S) by fluorescence polarization (FP) measurements in a static system using DPH (7).

In this study we have tested the performance of the FACS II cell sorter in FP measurements. Calibration solutions (fluorescein and fluoresceinated BSA) and Hoechst 33258-labeled cells were measured on the FACS, and results were compared with static measurements in a spectrofluorimeter. The usefulness of a pseudodepolar-

izer for normalization of the outputs of the two detectors on the FACS was investigated. Using F9 teratocarcinoma cells as a cell model system, we have investigated with flow cytophotometry the changes in FP of six different membrane probes upon cell differentiation.

MATERIALS AND METHODS

Cell Culture and Induction of Differentiation

F9 teratocarcinoma cells (a gift from Dr. H. Jakob, Inst. Pasteur, Paris, France) were cultured in Dulbecco's modified Eagle's medium plus 10% fetal calf serum (FCS), 100 U/ml penicillin, and 0.1 mg/ml streptomycin on plastic tissue culture vessels coated with 0.1% gelatin. The F9 cells were harvested by incubation for 5 min in 2 mM EDTA in Ca^{++}- and Mg^{++}-free phosphate-buffered saline (PBS). For induction of differentiation, cells were cultured on coated 10-cm dishes (10^5 cells/dish) and treated with 10^{-7} M retinoic acid (type XX, all trans; Sigma, St. Louis, MO) and 10^{-3} M dibutyryl-cyclic-AMP monosodium salt (Boehringer, Mannheim, FRG) for 4 days, with a change of growth medium containing inducers after 2 days (17). Cells were then grown in normal growth medium for another 2 days before analysis. To harvest differentiated cells they were treated first for 5 min with EDTA (2 mM) followed by an additional 5 min with trypsin (0.125% w/v). Undifferentiated control cells were treated in the same way. The cells were washed once with PBS before labeling with the fluorescent probes. Friend erythroleukemia cells (GM-86) (a gift from Dr. G. Bosman, Dept. of Physiology, State University Utrecht, The Netherlands) were used for calibration and testing of the FACS. These cells were cultured in suspension in RPMI medium supplemented with 10% FCS and antibiotics.

Fluorescent Labeling of Cells

The following fluorescent membrane probes were used: DPH (diphenyl hexatriene); TMA-DPH (1-(4-trimethylammonium-phenyl)-6-phenyl-1,3,5-hexatriene); and 12-AS, 9-AS, 6-AS, and 2-AS (anthroyloxystearate probes with the anthroyloxy group attached at different positions at the alkyl chain). All probes were from Molecular Probes, Inc. (Junction City, OR) except DPH, which was from Aldrich (Beerse, Belgium). Stock solutions of DPH (2 mM) and the AS probes (6 mM) were prepared in distilled tetrahydrofuran, while TMA-DPH (2 mM) was dissolved in dimethyl sulfoxide (DMSO). Solutions of AS probes were freshly prepared, while stock solution of DPH and TMA-DPH were stored at 4°C. The fluorescent probes were dispersed according to the method of Shinitzky and Barenholz (16) by adding 20 μl stock solution to 20 ml PBS under vigorous stirring. The cells were stained by adding 1 ml of dye dispersion to 1 ml cell suspension in PBS. ~2×10^6 cells). After incubation for 30 min at 37°C, the cells were centrifuged, suspended in ice-cold PBS, and stored on ice before measurement. The fluorescence of DPH- and TMA-DPH-stained cells was about 30 times higher than that of unstained cells; AS-stained cells were about eight times more fluorescent. Friend erythroleukemia cells were fixed for 30 min with 0.5% OsO_4, washed several times with PBS, and stored at 4°C. Staining with Hoechst 33258 (9) (1 μg/ml) was done for 1 h at room temperature.

Static FP Measurements on a Spectrofluorimeter

Measurements were done on a Perkin Elmer (Norwalk, CT) fluorimeter (type: MPF-44B) that had been equipped with adjustable polaroid polarizers (Melles Griot, Farnham, Surrey, England). Excitation was at 488 nm (3-nm slit) with emission at 530 nm (10-nm slit) for fluorescein or fluoresceinated BSA solutions, and at 375 nm (10-nm slit) with emission at 430 nm (20-nm slit) for Hoechst 33258-labeled Friend cells. Measurements were performed at room temperature. The fluorescent signals from Hoechst 33258-labeled cells were corrected afterward for the signals of unstained cells. Correction for polarization sensitivity of the emission detection system was done by measuring $I_\parallel$ and $I_\perp$ with horizontal (H) excitation. Polarization was determined by measuring with vertical (V) excitation and expressed as

$$\left(\frac{I_\parallel}{I_\perp}\right)_V \times \frac{1}{C};\ C \text{ being } \left(\frac{I_\parallel}{I_\perp}\right)_H$$

Polarization Measurements on the FACS II

Measurements were done at room temperature with a FACS II cell sorter (Becton Dickinson, Sunnyvale, CA) equipped with 2-W and 5-W argon ion lasers (164-01 and 164-05, Spectra Physics, Mountain View, CA) emitting, respectively, 200 mW 488 nm and 40 mW UV (351.1 nm and 363.8 nm) light. A minor modification was made in in the electronics of the FACS to extend the output of the analog signal divider to make more profitable use of the 256 channels of the ND-100 multichannel analyzer (Nuclear Data, Schaumburg, IL). Figure 1 shows a diagram of the optical system, indicating the extra components for polarization measurements. Barrier filters (Fig. 1:3) were placed in the filter tube just behind the objective lens. The following barrier filters were used: a 530-nm interference filter, half-bandwidth 10 nm (Balzers AG., Balzer, Liechtenstein) (488-nm excitation for calibration solutions of fluorescein and fluoresceinated BSA) or a KV 400 and a K 420 filter (Schott, Mannheim, FRG). (UV excitation for solutions of methylumbelliferon and all single-cell measurements). When depolarization of fluorescent light was needed (see below), a pseudodepolarizer (Cornu type, Melles Griot, Farnham, Surrey, England) was inserted in a filter holder and placed in front of the beam splitter (Fig. 1:4), the orientation being such that the plane of the inner surface where the two halves of the prism are cemented was perpendicular to the XY plane (Fig. 1). The standard 50/50 beamsplitter (Fig. 1:5) was used to divided the fluorescent light. Rotatable polaroid filters (PF) (Fig. 1:6, 7) as provided with the FACS II equipment were placed in front of the two photomultipliers (PM1 and PM2).

Static polarization measurements on FACS. For determination of FP of calibration solutions, static mea-

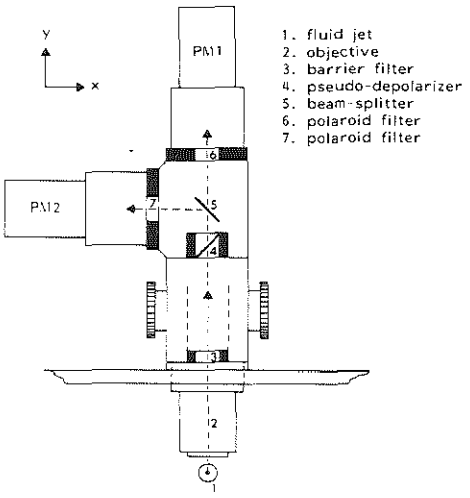

Fig. 1. Diagram of the optical system of the FACS II, indicating the extra components needed for fluorescence polarization measurements.

surements were done with PM1 (Fig. 1) only, rotating the PF (Fig. 1:6) for measurement of parallel and perpendicular components. Anode current was converted to a voltage by way of an operational amplifier and measured with a voltmeter.

In the single-cell experiments where a pseudodepolarizer was used to depolarize the fluorescent light (see below), the position of the objective lens (Fig. 1:2) relative to the pseudodepolarizer was adjusted in such a way that the fluorescent light beam covered the entire area of the pseudodepolarizer. This was done using a solution of methylumbelliferon (0.75 mg/ml) as a test sample, by moving the lens from the optimum position (maximum fluorescence intensity) toward the fluid jet till a fluorescence signal of 0.9 times the maximum signal was reached. This procedure was established experimentally and results in a fluorescent light beam covering slightly more than the area of the pseudodepolarizer.

Single-Cell Polarization Measurements

For single-cell measurements the beam splitter was inserted and PF were turned in such a way that the perpendicular component ($I\perp$) was measured with PM1 and the parallel component ($I\parallel$) with PM2 (Fig. 1). The gains of the two amplifiers were set at the same level, and the cells were analyzed according to the following procedure:

1. The pseudodepolarizer was inserted to depolarize the fluorescent light ($I\parallel = I\perp$), and the PM outputs were equalized by adjusting the PM voltages.
2. After removal of the depolarizer, the cells were measured again. The signals from the two PM were divided with the ratio circuit of the FACS and collected with the ND-100 multichannel analyzer. The channel number (± 1 channel) of the maximum of the distribution was estimated with one of the markers of the ND-100.

Channel numbers were converted into ratios $I\parallel/I\perp$ by means of a calibration line. This calibration line was made (while measuring fluorescent cells) by using the signal of one photomultiplier and feeding it into the two amplifiers at the same time. At equal amplifier setting, the ratio of the outputs of the two amplifiers is 1; by changing the coarse gains, the ratio can be changed to 2 or ½. Measurement of these signals, through the ratio circuit, provided the (straight) calibration line.

RESULTS

Testing of FACS

The various components of our FACS II cell sorter were tested with regard to their performance in fluorescence polarization measurements. To test for possible aberrations caused by the optical system, static measurements on the FACS using calibration solutions of fluorescein (10^{-6} M) and fluoresceinated-BSA (0.05 mg/ml) (8), were compared with measurements on a Perkin Elmer spectrofluorimeter. In the Perkin Elmer values $I\parallel/I\perp$ of 1.03 and 1.92 were found for fluorescein and fluoresceinated BSA, respectively, while with the FACS values of 1.04 and 1.93 were obtained. The usefulness of the pseudodepolarizer (used for normalization of the two photomultiplier signals) was demonstrated using the fluoresceinated BSA. Insertion of the pseudodepolarizer in the fluorescence channel changed the ratio $I\parallel/I\perp$ from 1.93 to 1.00, indicating effective depolarization of the fluorescent light. The overall performance of our FACS II cell sorter (optics and electronics) when measuring fluorescence polarization of single cells using UV excitation (also needed for the membrane probes we have used) was tested using Hoechst 33258-labeled Friend cells as a model system. Measurements on the FACS gave a mean value for $I\parallel/I\perp$ of 2.40 (number of experiments (n) = 12, SD = 0.05). Static measurements on the Perkin Elmer also gave a value of 2.40 (anisotropy value r = 0.32).

Fluorescence Polarization of DPH and TMA-DPH in Undifferentiated and Differentiated F9 Teratocarcinoma Cells

F9 teratocarcinoma cells were induced to differentiate by treatment with retinoic acid and dibutyryl-cAMP as described in Materials and Methods. Control cells without any additions were cultured in parallel. Cells were stained with DPH or TMA-DPH and polarized emission of single cells was measured on the FACS II. Using DPH mean values of $I\parallel/I\perp = 1.55$ (SD = 0.05, n = 8) for control cells and 1.74 (SD = 0.09, n = 7) for differentiated cells were obtained. Thus a mean increase of 0.19 (range 0.10–0.29) was found. Using TMA-DPH these

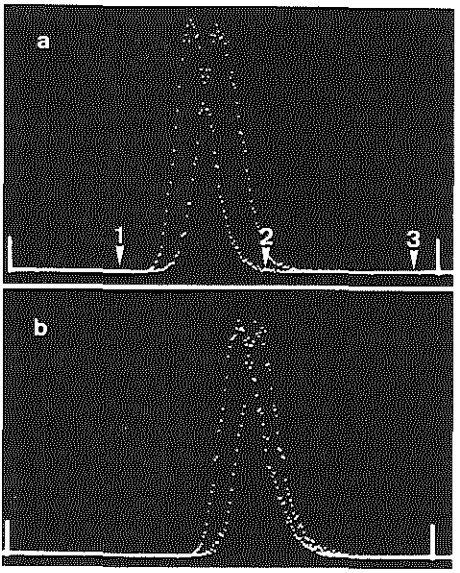

Fig. 2. Fluorescence polarization of DPH and TMA-DPH in undifferentiated and differentiated F9 teratocarcinoma cells. x-Axis: degree of polarization (ratio I‖/I⊥); ratios 1, 2, and 3 are indicated in a. y-Axis: number of cells (linear scale). a) DPH, undifferentiated cells, peak value 1.48; differentiated cells, peak value 1.67. b) TMA-DPH, undifferentiated cells, peak value 1.82; differentiated cells, peak value 1.95.

values were 1.87 (SD = 0.06, n = 6) for control and 2.02 (SD = 0.06, n = 6) for differentiated cells. Thus with this probe a mean increase of 0.15 (range 0.12–0.18) was found after differentiation (see Fig. 2).

As observed with the fluorescence microscope, the cells stained with DPH were evenly stained (the nucleus could be seen as an unstained region), while at the outer membrane of the cells brightly fluorescent spots were seen. Cells stained with TMA-DPH showed some internal labeling, but plasma membrane labeling was often present as a fluorescent ring around the cells. No fluorescent spots were seen. No large differences in staining of undifferentiated and differentiated cells were observed, but detailed comparison was not possible due to rapid fading of the fluorescence.

FP of Anthroyloxystearate Probes

The model system of undifferentiated and differentiated F9 cells was used to test the ability of a set of four different anthroyloxystearate probes to sense the changes found with DPH and TMA-DPH. The results of one experiment are shown in Figure 3c–f. Virtually no increases in fluorescence polarization were found with 12-AS, 9-AS, 6-AS, and 2-AS. In that same experiment the differences seen when cells were stained with DPH or TMA-DPH are shown in Figure 3a and b. As observed with the fluorescence microscope, most fluorescence came from the interior of the cells using the AS probes.

DISCUSSION

The aim of this study was to investigate the ability of six different fluorescent membrane probes to sense changes in fluidity of membranes of living cells by flow cytometry. Correct measurement of FP in a flow system may be complicated by a number of optical and electronical factors (1, 8). We therefore tested the performance of the FACS II cell sorter with respect to these measurements. Calibration solutions of fluorescein (nearly completely depolarized) and fluoresceinated BSA (fairly polarized) gave FP values on the FACS nearly identical to values obtained with a conventional fluorimeter, indicating that none of the optical components effect the polarization of the fluorescent light. On theoretical grounds, depolarization of the signal (from 1.93 to 1.84) is expected, caused by the large numerical aperture of the objective lens (8, 10). In practice the depolarization may be less because part of the fluorescent light is obscured by the bar as well as by the nozzle, favoring relatively more light from the central part of the emitted light beam.

For measurements on single cells the signals from the two photomultipliers detecting, respectively, I‖ an I⊥ have to be normalized by measuring a signal corresponding to totally depolarized emission. We found that a pseudodepolarizer could be used to depolarize emitted light from I‖/I ⊥ = 1.93 to 1.00. The good performance of this device is caused by the relatively low degree of polarization of fluorescent light (theoretical maximum, 3).

Two other simple devices are available for equalizing I‖ and I ⊥. First, the direction of polarization of the laser light may be turned 90° by means of a ½ λ retardation plate. Since most retardation plates will function exactly for one wavelength only, separate plates have to be used for different excitation wavelengths. This also poses a problem with UV excitation, where two wavelengths are simultaneously used (351.1 nm and 363.8 nm). Second, it is possible to insert a third polarization filter in front of the beam splitter orientated at 45° with respect to both analyzers, but this requries very careful orientation of the three polarization filters with respect to each other. Therefore the use of the pseudodepolarizer has distinct advantages, because it can be used regardless of the excitation wavelength, and it does not require the extremely accurate alignment of the analyzers.

When overall performance of the cell sorter, optics as well as electronics, was tested using Hoechst 33258-stained cells, the mean value obtained was the same as with the static measurements on the spectrofluorimeter (I‖/I ⊥ = 2.40, r = 0.32) and in agreement with the value of 0.32 reported in the literature (8). Since different

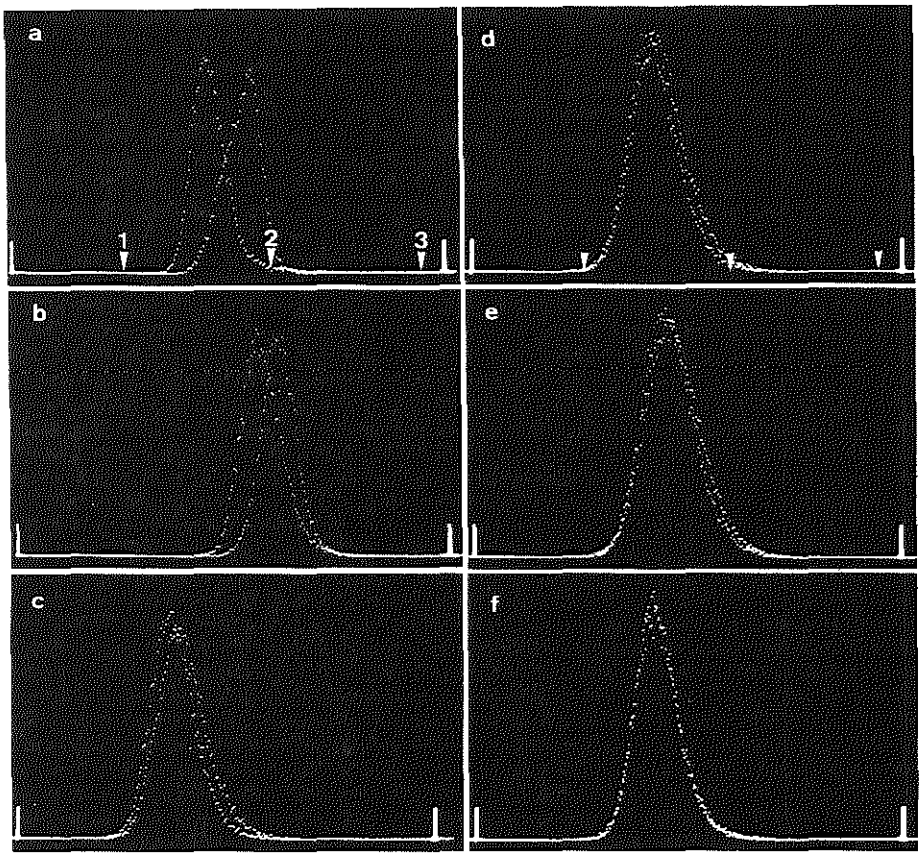

Fig. 3. Fluorescence polarization of anthroyloxystearate probes (12-AS, 9-AS, 6-AS, and 2-AS) in undifferentiated and differentiated F9 cells, compared with DPH and TMA-DPH: x-axis, degree of polarization (ratio $I_{\parallel}/I_{\perp}$)—ratios 1, 2, and 3 are indicated in a and d; y-axis, number of cells (linear scale). a) DPH, undifferentiated cells, peak value 1.55; differentiated cells, 1.84. b) TMA-DPH, undiff. 1.86; diff. 2.00. c) 12-AS, undiff. 1.31; diff. 1.37. d) 9-AS, undiff. 1.43; diff. 1.48. e) 6-AS, undiff. 1.55; diff. 1.58. f) 2-AS, undiff. 1.46; diff. 1.47.

independent measurements showed some variation (SD = 0.05, n = 12; range 2.32–2.47) the use of some form of internal standard will be necessary for comparison of different experiments.

Relatively large increases in FP were seen with teratocarcinoma cell differentiation using DPH and TMA-DPH. From the figures it can be seen that upon differentiation all cells shifted toward higher polarization values with no appreciable widening of the curves. Although some of the curves after differentiation were not completely Gaussian (for example, Fig. 3a) this was not found reproducibly for independent inductions. Thus these single-cell polarization measurements show that the major effect was a shift of all cells toward higher FP

values with very little heterogeneity between the cells. For this reason also we used the shift of the maximum of the distribution as a measure of the change.

The increase in fluorescence polarization was found when cells were treated with 10^{-7} M RA and 10^{-3} M cAMP for 4 days followed by another 2 days in the absence of the inducers. With cAMP (10^{-3} M) alone no increase in fluorescence polarization was found (results not shown). Although morphological differentiation was observed after treatment with 10^{-7} M RA alone, in most experiments no increase in FP was observed (results not shown). Thus it appears that the increase in FP occurs later than morphological differentiation.

From our values for DPH polarization $I_{\parallel}/I_{\perp} = 1.55$ and 1.74 for undifferentiated and differentiated cells, respectively; values for "apparent" microviscosity of 1.82 and 2.96 Poise may be calculated according to (16). These values are very similar to those found by Jetten et al. (7) for RA-induced OC15S teratocarcinoma cells.

The increase in FP seen with DPH and TMA-DPH may have been caused by the increase in cholesterol content of the plasma membrane upon differentiation of F9 cells as reported by Searls and Edidin (14). However, interpretation of FP changes in terms of biological parameters is severely complicated when the fluorescent probes label multiple cellular compartments (2, 3, 6). A change can then be caused by a change in any of the different compartments as well as by a change in the relative volumes of the compartments if these have different FP values. With DPH the cells were labeled completely, and brightly fluorescent spots were also observed. In addition, the rapid fading of the fluorescence (15) did not permit detailed comparison of undifferentiated and differentiated cells. Since TMA-DPH carries a charged group, the intracellular penetration would be more difficult for this probe than for DPH. Although some intracellular fluorescence was still present with this probe, definite plasma membrane labeling was also seen. The increase in FP seen upon differentiation of the F9 cells demonstrates its usefulness for this type of polarization measurement. Further improvement of the staining protocol, e.g., by incubation at low temperature, may possibly eliminate internal labeling.

Virtually no changes in FP were seen with the AS-probes, although three of these probes (6-AS, 9-AS, and 12-AS) have been observed to be applicable in measurements of FP changes in membrane model systems (18, 19). Our microscopical observations that the AS probes were localized mainly inside the cells may explain why no change in FP was observed.

In conclusion, the experiments demonstrate that reliable FP measurements can be made with the FACS II cell sorter. Also, the usefulness of the teratocarcinoma cell system for the evaluation of the behavior of membrane probes in intact living cells is demonstrated, and these analyses showed that, in addition to DPH, TMA-DPH can be used for this type of experiment. Finally, differentiation induction of undifferentiated F9-cells caused an increase in FP in the whole population of undifferentiated cells. This increase occurred as a late event during this induction, after morphological differentiation.

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PAPER 7

Gene expression in flow sorted mouse teratocarcinoma × human fibroblast heterokaryons

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Abstract. Mouse teratocarcinoma cells and primary human fibroblasts were fluorescently labelled with fluorescein isothiocyanate (FITC)- and trimethylrhodamine isothiocyanate (TRITC)-stearylamine respectively. After fusion populations highly enriched for red-green heterokaryons (around 80%) were isolated from the fusion mixture using a FACS II cell sorter.

To study gene expression in the early hybrids [^{35}S] methionine-labelled proteins synthesized by the sorted cells at two and three days after fusion were analysed by two-dimensional gel electrophoresis. Three spots were denser in gels of the fused cells than in those of 1:1 mixtures of parental cells. For one of these proteins it could be demonstrated that this reflects the enhanced synthesis of a mouse-specific protein present only in small amounts in teratocarcinoma cells. All three proteins were synthesized in relatively large amounts by differentiated mouse cells.

Collagen (type I) synthesis by the sorted hybrid cells was studied by analysing the [^{3}H] proline-labelled material secreted into the medium. Analysis by sodium dodecyl sulphate (SDS)-gel electrophoresis and two-dimensional non-equilibrium pH gradient electrophoresis showed that the material secreted by the fused cells five days after fusion was the same as that secreted by the human fibroblasts. No evidence was obtained for synthesis of mouse α2(I) collagen. The amount of collagen produced by the sorted cells five days after fusion was about half the amount produced by the human fibroblasts. Immunofluorescence studies also showed that collagen synthesis was not suppressed after fusion both in heterokaryons and synkaryons.

In conclusion, we did not find evidence for activation of a previously completely silent mouse gene in the fused cells. The results show, however, that the fused cells do resemble the differentiated fibroblasts rather than the undifferentiated teratocarcinoma cells.

Introduction

In studies on gene regulation and cell differentiation both hybrid cells and heterokaryons are employed [for review see 28]. The use of heterokaryons (the initial products after cell fusion) has advantages over the use of proliferating hybrid cells since analysis is performed before chromosomal losses and possible selection have occurred. The heterokaryon system has been employed in only a few studies, mainly due to technical difficulties associated with the analysis of heterokaryons against a background of unfused cells. One approach is to perform analysis at the single cell level [e.g. 22]. Another possibility is to isolate the heterokaryons from the fusion mixture, which can be done by flow sorting [15–17].

In most experiments crosses between two different differentiated cells have been studied. It might be advantageous, however, to use as one parent an undifferentiated teratocarcinoma cell. These cells are thought to correspond to the cells of the early embryo and retain the capacity to differentiate [for review see 21]. Therefore they may be especially useful in cell fusion studies to elucidate the existence of factors, possibly present in differentiated cells, positively regulating gene expression.

In this study we have fused PCC4 AZA1 mouse teratocarcinoma cells with primary human fibroblasts and purified the heterokaryons by flow sorting. The proteins synthesized by the sorted cells were analysed by two-dimensional gel electrophoresis [23] to see whether activation of mouse proteins not present in the teratocarcinoma cells would occur. Furthermore we have studied the production of type I collagen, a specific product synthesized and secreted by fibroblasts [13] but not by undifferentiated teratocarcinoma cells [2, 8, 27, 31], to see whether synthesis would continue and whether activation of mouse collagen type I would occur.

Methods

Cell culture, fusion, and heterokaryon isolation

Mouse teratocarcinoma cells PCC4 AZA1, mouse 3T3 fibroblasts, and primary human fibroblasts isolated in this laboratory (80RD174) were cultured in Ham's F10 medium supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 μg/ml). Before cell fusion cells were cultured overnight in medium buffered at pH = 8.0 [7], supplemented with fluorescent stearylamines [15, 17] (PCC4 cells, FITC-stearylamine; fibroblasts, TRITC-stearylamine; final concentration 0.25 vol % diluted stock; in water these dilutions gave an absorption of: $A_{470} = 0.0425$ (FITC-stearylamine) and $A_{550} = 0.085$ (TRITC-stearylamine)). Cells were fused at pH 8.0 using inactivated Sendai virus [14] (500–1000 HAU/ml) using excess PCC4 cells (ratio PCC4/fibroblasts approximately 4/1; total amount of cells up to 20×10^6 in 1 ml fusion volume). Fused cells were replated and cultured for about 5 h at

pH 8.0. They were trypsinized by treatment with trypsin/EDTA (0.1 g/l trypsin, 0.04 g/l EDTA) for 5 min (to detach PCC4 cells) and 0.125% w/v trypsin for another 5 min (to detach fibroblasts). Flow analysis and sorting was done with a FACS II cell sorter (Becton and Dickinson) as described before [15, 16]. For determination of the purity of the sorted heterokaryon fraction, samples were cultured overnight on glass coverslips in Nunc 4 well plates (Nunc, Denmark) fixed with 10% (w/v) formol in phosphate buffered saline (PBS) followed by methanol/acetic acid (3:1 v/v) and stained with Hoechst 33258. With fluorescence microscopy nuclei of mouse origin can be distinguished by the presence of chromocentra [5, 22].

Two-dimensional-electrophoresis of [^{35}S] labelled proteins

Cells were cultured in normal growth medium, whereafter they were labelled with [^{35}S] methionine (50 µCi/ml; sp. act. >600 Ci/mmol, Radiochemical Centre, Amersham, England) for 24 h. About 10,000 cells gave sufficient material for one gel (250,000 trichloroacetic acid (TCA) precipitable cpm/gel). Two-dimensional gel electrophoresis was done essentially according to the method of O'Farrell and O'Farrell [23], as described before [29].

Extraction of labelled collagen

Sorted cells were cultured in chemical selection medium (normal growth medium with hypoxanthine (10^{-4} M), aminopterine (4×10^{-7} M), and thymidine (1.6×10^{-5} M) (HAT) [20] and ouabain (10^{-6} M) before labelling. Cells were labelled in proline-free F10 or Dulbecco's modified Eagle medium supplemented with 10% dialysed FCS, antibiotics, 0.3 mM sodium ascorbate, and 50 µg/ml β-aminopropionitrile (Aldrich, Beerse, Belgium) [11]. [^{3}H] proline (25–50 µCi/ml, sp. act. 18 or 21 Ci/mmol, Radiochemical Centre, Amersham) was added for 24 h and collagen secreted into the medium was extracted by a procedure based on previous research [4, 12, 32]. Briefly: the medium was acidified with acetic acid till 0.5 M and after centrifugation (Eppendorf minifuge, 10 min, 4° C) further acidified till 1 M acetic acid and treated overnight with pepsin (2 mg/ml, at room temperature). Unincorporated label was removed using Sephadex columns as described by Penefsky [25], and the material was lyophilised.

Qualitative analysis of collagens by gel electrophoresis

For SDS-gel electrophoresis according to the method of Laemmli [19] the lyophilised material was dissolved in SDS-sample buffer and analysed on 5% gels. For collagenase treatment the lyophilised material was dissolved in 0.05 M TES (N-tris (hydroxymethyl) methyl-2-aminomethane sulphonic acid), 0.35 mM $CaCl_2$, pH 7.5, and treated with collagenase (Worthington CPSLA, 0.1 mg/ml) for 5 h at 37° C, whereafter twice concentrated sample buffer was added. Collagenase was used without further purification and may therefore have contained other proteases [26]. Two-dimensional gel electrophoresis with non-equilibrium pH gradient electrophoresis (NEPHGE) was done essentially according to the method of O'Farrell et al. [24]. Ampholines (LKB) were in the range 5–8 (1.6% w/v) and 3.5–10(0.4% w/v), electrophoresis in the first dimension was for 1200 V × h, and a 5% gel was used in the second dimension.

Quantitative analysis of collagen synthesis

Human fibroblasts (10^5), PCC4 cells (0.5×10^5), and sorted heterokaryons (10^5) were plated in 24-well tissue culture plates and cultured for four days (PCC4 cells and fibroblasts in normal growth medium and the heterokaryons in selective medium (see above)). Incubation with [^{3}H] proline and extraction of collagen was done as described above. After passage through the Sephadex columns (see above) the amount of TCA precipitable counts (10% TCA and 10 µg/ml bovine serum albumin (BSA) carrier) before and after collagenase treatment (see above) was determined by liquid scintillation counting. For protein determination the cell layers were washed twice with PBS and air dried. Cells

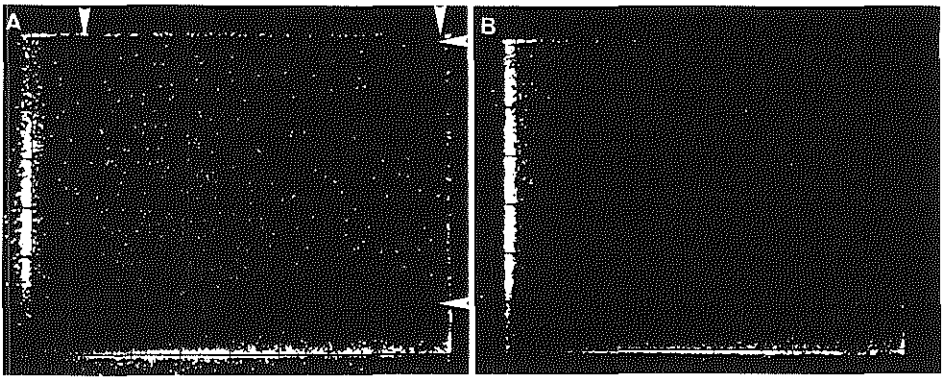

Fig. 1 A, B. Flow analysis of a fusion mixture of green fluorescent labelled teratocarcinoma cells fused with red fluorescent labelled human fibroblasts (**A**) and a mixture of unfused cells (**B**). *X-axis:* green fluorescence (a.u.), *Y-axis:* red fluorescence (a.u.), 4000 cells were analysed. *Arrowheads* indicate sorting window

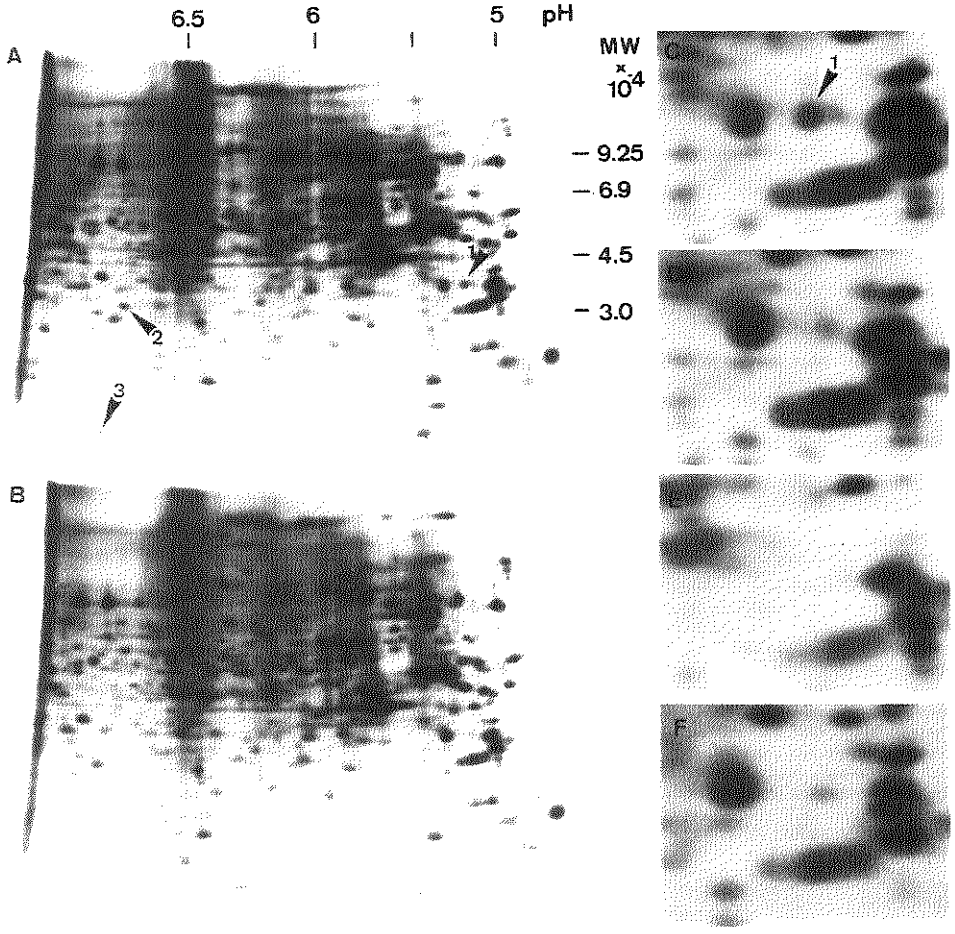

Fig. 2A–F. Two-dimensional patterns of radiolabelled proteins. **A** Sorted cells from a fusion mixture (see Fig. 1A) analysed 48 h after fusion. A parallel culture sorted and fixed at the same time and stained with Hoechst showed the presence of 16.5% heterokaryons, 47% synkaryons, 9% PCC4 cells, and 28% human fibroblasts. **B** 1:1 mixture of labelled proteins from PCC4 cells and human fibroblasts. *Arrowheads* in **A** indicate spots denser in **A** than in **B**, **C**, and **D**: enlarged region from **A** and **B** respectively. **E** and **F** The same regions from separate gels of PCC4 cells and human fibroblasts respectively

were solubilised with 100 µl 0.02 M NaOH and stored at −20° C. Protein was determined using fluorescamine [6] as described before [18].

Immunofluorescence

Cells cultured on glass coverslips were washed with PBS, air dried, and postfixed with acetone [9]. Immunofluorescence staining was done using a sheep anti-human type I collagen antiserum (a kind gift from Dr. E. Solomon, Imperial Cancer Fund, London and Dr. B. Sykes, Oxford University), and with FITC-conjugated rabbit anti-sheep immunoglobulin serum (Dakopatts, Denmark). Under our experimental conditions the antiserum also reacts with collagen in mouse 3T3 cells. After staining with Hoechst (see above) cells were used for fluorescence microscopy. Treatment with cycloheximide (20 µg/ml) was for 4 h prior to fixation. For analysis of resumption of collagen synthesis the inhibitor was washed away and cells were fixed after a further culture period of 16 h.

Results

Flow sorting of fused cells

Green fluorescent mouse teratocarcinoma cells and red fluorescent human fibroblasts were fused using inactivated

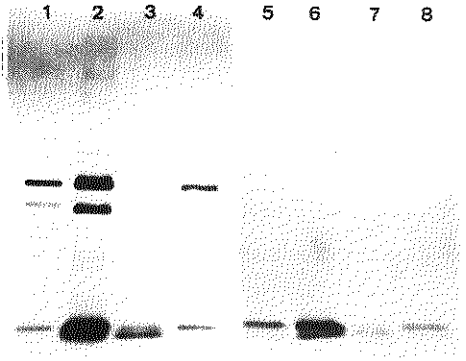

Fig. 3. SDS-gel electrophoresis of [^{3}H] proline-labelled pepsin-resistant polypeptides extracted from the culture medium. Migration from top to bottom. Lanes 1, 2, 3, and 4 respectively human fibroblasts, mouse 3T3 fibroblasts, PCC4 cells, and sorted fused cells analysed five days after fusion (Hoechst analysis of a parallel culture showed that 90% of the cells were heterokaryons and synkaryons) lanes 5, 6, 7, and 8: The same cells but samples were treated with bacterial collagenase

Sendai virus, and heterokaryons were isolated from the fusion mixture by flow sorting. Maximally 8% of the cells fell within the sorting window (Fig. 1). The percentage of fused cells in the sorted cell fraction was determined after overnight cultivation by staining with Hoechst dye. As indicated by this analysis purification till around 80% fused cells could be obtained (six experiments: 63, 84, 81, 79, 76, 87%). At this time (about 24 h after fusion) synkaryons (fused cells in which the nuclei had also fused) were already identified. Three days after fusion synkaryons made up about 70% of all fused cells, indicating rapid synkaryon formation.

Two-dimensional protein pattern of sorted fused cells

In two independent experiments fused cells were sorted and labelled with [^{35}S] methionine for a period of 24 h, at respectively 24 and 48 h after fusion. The pattern of the synthesized proteins was analysed by two-dimensional gel electrophoresis, and compared with that of a 1:1 mixture of parental cells. Duplicate gels were run for each sample and a result for the 24–48 h labelling period is shown in Fig. 2A, B. When all gels were compared three spots were found to be denser in the gels of the fused cells than in those of the mixtures (Fig. 2A, 1, 2, and 3). The largest difference

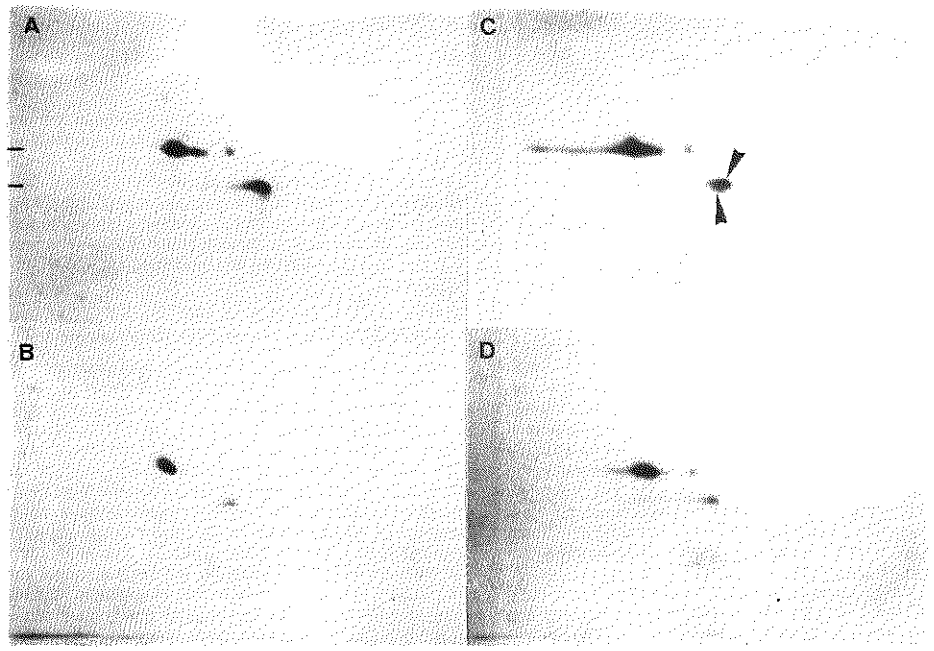

Fig. 4A–D. Nonequilibrium pH gradient two-dimensional gel electrophoresis of [^{3}H] proline-labelled pepsin resistant polypeptides extracted from the culture medium. **A** Human fibroblasts, the position of α1(I) and α2(I) collagen in the second dimension (see Fig. 3) is indicated. **B** Mouse 3T3 fibroblasts, **C** mixture of human and mouse fibroblasts. Note the different mobilities of the mouse and human α_2(I) chains (*arrowheads*). **D** Sorted fused cells analysed five days after fusion (see Fig. 3)

Table 1. Synthesis of collagenous polypeptides

Cells	Total protein (μg)	Total pepsin[a] resistant radioactivity (cpm)	Collagenase resistant radioactivity (cpm)	Collagenase sensitive radioactivity (cpm)	(cpm/μg protein)
Fused[b] cells	48±1[c]	28,000±1,000[c]	8,000±1,200[c]	20,000	417
	76±1	20,000±3,000	2,500±2,000	17,500	230
Human fibroblasts	80±16	63,400±3,100	9,300±1,000	54,100	680
PCC4 AZA1	37±2	1,450±140	3,280±440	–	–

[a] Cells were labelled with [^{3}H] proline for 24 h
[b] Sorted cells analysed five days after fusion (two separate cultures). Hoechst analysis of a parallel culture indicated 97% heterokaryons and synkaryons at the end of the culture period
[c] Mean of duplicate determinations

Table 2. Intracellular presence of collagen[a]

Treatment of cells	Fibroblasts[b]		PCC4 AZA_1[b]		Heterokaryons[b]		Synkaryons[b]	
	%+	%−	%+	%−	%+	%−	%+	%−
None	98 (57)[c]	2 (1)	0	100 (164)	61 (14)	39 (9)	82 (32)	18 (7)
Cycloheximide 4 h	22 (5)	78 (18)	0	100 (25)	0	100 (9)	22 (4)	78 (14)
Cycloheximide + 20 h recovery	98 (48)	2 (1)	0	100	29 (6)	71 (15)	63 (17)	37 (10)

[a] Intracellular collagen demonstrated by immunofluorescence using α-type I collagen antiserum
[b] Cells identified by Hoechst staining and morphology
[c] Number in parenthesis gives number of cells counted

in density was found for spot 1, which was not visible in the gels of the mixtures. However, when proteins of the parental cells were analysed separately this protein could be detected in small amounts in gels of PCC4 cells (Fig. 2E), but not in gels of the human fibroblasts (Fig. 2F). Spot 2 was found with decreased density both in gels of teratocarcinoma cells and of human fibroblasts, while spot 3 was observed in low density only in the teratocarcinoma gels (results not shown). No other reproducible differences between the pattern of the fused cells and the mixtures could be observed.

Collagen synthesis in fused cells

For the qualitative analysis of collagen synthesis, heterokaryons were isolated, cultured, and analysed five days after fusion. Chemical selection was applied from 24 to 96 h after fusion to kill unfused human fibroblasts and PCC4 cells. After labelling with [^{3}H] proline for 24 h the collagenous proteins were extracted from the medium. Analysis by SDS-gel electrophoresis showed two collagenase sensitive bands in medium samples from the sorted cells (Fig. 3 lane 4). At that position the major bands in medium samples from the human fibroblasts (Fig. 3 lane 1) and mouse 3T3 fibroblasts (Fig. 3 lane 2) were also present. These bands correspond to the pepsin-resistant parts of α1(I) and α2(I) collagen [1, 12]. In samples from PCC4 cells these bands were not detected (Fig. 3 lane 3). Two distinguish between human and mouse collagens two-dimensional nonequilibrium pH gradient gel electrophoresis was performed (Fig. 4). The pattern obtained for the proteins from the medium of the fused cells (Fig. 4D) resembled that of the human fibroblasts (Fig. 4A). Since human- and mouse α2(I) collagen differed in electrophoretic mobility (Fig. 4C), these results therefore give no indication of synthesis of mouse α2(I) collagen in the fused cells.

The amount of pepsin-resistant collagenous proteins synthesized and secreted by the fused cells was determined at five days after fusion and compared with the amount produced by fibroblasts and PCC4 cells (Table 1). The fused cells still secreted appreciable amounts of collagenous material (about 320 cpm/24 h μg protein) comparable with the amount produced by the human fibroblasts (680 cpm/24 h μg protein), while no synthesis was detected for the PCC4 cells.

Immunofluorescent detection of intracellular collagen

Fused cells were isolated by flow sorting and analysed three days after fusion by immunofluorescence using an antiserum against type I collagen (Table 2). No chemical selection was applied and human fibroblasts and PCC4 cells also present served as controls. Most heterokaryons and synkaryons showed a granular intracellular staining (74%), like the human fibroblasts, while the teratocarcinoma cells were negative. Treatment with cycloheximide resulted in disappearance of the fluorescence, and reappearance occurred after removal of the inhibitor (Table 2). This shows that the collagen was still secreted by the heterokaryons and synkaryons, and therefore that the intracellular collagen detected reflects de novo synthesis of this protein at the time of analysis. In an independent experiment the percentage of heterokaryons and synkaryons positive in the immunofluorescence test was 83% at three and 79% at five days after fusion. These experiments show that collagen synthesis continued in the heterokaryons and synkaryons,

and confirm the results obtained by the radioactive assay (see above).

Discussion

In order to study gene expression in heterokaryons between mouse teratocarcinoma cells and human fibroblasts, we have isolated these cells from the mixture of cells after fusion using a previously described technique [15–17]. A purification till around 80% fused cells could be obtained in this cross. The relative amount of heterokaryons decreased and the amount of synkaryons (cells with fused nuclei) rapidly increased till about 70% already three days after fusion. Therefore in our experiments all analyses were performed on mixtures of heterokaryons and synkaryons.

The protein pattern of the sorted fused cells was analysed by two-dimensional gel electrophoresis. In this way induction of expression in the fused cells of a gene initially expressed only by one of the parental cells may be detected if a difference in isoelectric point exists between the human- and mouse protein. Since we look for a *new* protein, the presence of a small amount of parental cells will not interfere with the analysis. Therefore in order to avoid possible disturbances in the two-dimensional gel pattern, no further chemical selection was applied and proliferation of the initially about 20% of contaminating unfused cells after sorting was allowed. Three spots were denser in the gels of the fused cells than in those of mixtures of parental cells. Spot 1 was not observed at all in the gels of the mixtures but inspection of gels of the separate parental cells showed that this protein could be detected in small amounts in the teratocarcinoma cells. This protein has also been found as a dense spot in two-dimensional gels of differentiated mouse endoderm cells [28] and of mouse fibroblasts (results not shown), but not in gels of human fibroblasts. The human fibroblasts produce a relatively large amount of a protein with a slightly higher isoelectric point than the induced protein 1, which is absent in gels of the differentiated mouse cells and the mouse teratocarcinoma cells. Therefore the appearance of spot 1 in the fused cells most probably reflects the enhanced synthesis of a mouse protein present in relatively large amounts in differentiated mouse cells which has an equivalent with a slightly different isoelectric point in differentiated human fibroblasts. No such conclusion can be drawn for proteins 2 and 3. Protein 2 is synthesized already by both parental cells, while protein 3, which is synthesized only by the teratocarcinoma cells, does not have a human equivalent (results not shown). However, like spot 1 both protein 2 and 3 are found as dense spots on gels of differentiated mouse endoderm cells and mouse fibroblasts (results not shown). Although the spots are clearly not specific for fibroblast differentiation only, all together the two-dimensional pattern indicates that the fused cells resemble the differentiated fibroblast rather than the undifferentiated teratocarcinoma cell.

Collagen synthesis and secretion were also studied in the fused cells. Fibroblasts synthesize and secrete relatively large amounts of collagen mainly of type I (about 80%) and smaller amounts of collagen type III (about 20%) [3, 11]. Undifferentiated mouse teratocarcinoma cells synthesize only very small amounts of type IV collagen [2, 27, 31], while upon differentiation in some cases the induction of type I collagen has been demonstrated [2, 8]. Thus collagen type I is a suitable specific marker in our cell system. In these experiments chemical selection was applied after sorting resulting in a very high percentage of fused cells (up to 97%), which allowed us also to determine whether extinction of collagen synthesis would occur in the fused cells. The electrophoretic analysis of the pepsin resistant [^{3}H] proline labelled material secreted by the sorted fused cells five days after fusion showed two collagenase-sensitive bands on SDS-gels, with mobilities expected for α1(I) and α2(I) collagen chains [1, 11]. Any α1(III) collagen would not be detected separately since this has a mobility similar to that of the α1(I) chains [11]. A small but reproducible difference in mobility for the α2(I) chains of mouse 3T3 cells and human fibroblasts was found on nonequilibrium two-dimensional gels. Since the mouse α2(I) spot was not detected in the pattern obtained for the fused cells, this indicates that no appreciable activation of the mouse α2(I) gene occurs after fusion. The human and mouse fibroblasts each have a different small extra spot with approximately the mol. wt. of α1(I), while the fused cells only show the spot seen with the human fibroblasts. Thus in all these aspects the fused cells are identical to the human fibroblasts. Quantitative analysis showed that the amount of collagen produced by the fused cells five days after fusion was approximately half the amount synthesized by the human fibroblasts. This result was confirmed by the immunofluorescence studies, which also showed that collagen type I synthesis continued both in the heterokaryons and synkaryons. The estimated half-life for chicken type I collagen mRNAs is in the order of 10 h [30]. Using this datum, our finding that collagen synthesis still continued in the fused cells five days after fusion shows that collagen (I) mRNA production is not suppressed in the fused cells.

Because mouse teratocarcinoma cells correspond to very early undifferentiated cells [21], the use of these cells in cell hybridisation studies may be advantageous. However, experiments with proliferating hybrids between teratocarcinoma and differentiated cells have not revealed a simple pattern of dominance of either the teratocarcinoma – or the differentiated phenotype [10]. No studies on gene expression in heterokaryons using teratocarcinoma cells as one of the parental cells have been reported. However, in other crosses the existence of both activating [5, 33] and suppressing factors [22] have clearly been demonstrated. As a simple hypothesis one might expect undifferentiated teratocarcinoma cells to be more susceptible to activating factors than differentiated cells, while on the other hand they might lack suppressing factors. Since no activation of mouse α2(I) collagen was found in the fused cells and spot 1 in the two-dimensional gels reflects enhanced synthesis of a protein already expressed in small amounts by the teratocarcinoma cells, this study gives no evidence for factors, supplied by the fibroblasts, capable of activating completely silent teratocarcinoma genes. The teratocarcinoma cells, on the other hand, did not suppress collagen synthesis of the human fibroblasts and also the two-dimensional gel pattern suggests that the fused cells resemble the differentiated fibroblasts.

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