X-CHROMOSOME ACTIVITY AND EMBRYONAL CARCINOMA CELLS

by

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ABSTRACT

Embryonal carcinoma (EC) are the stem cells of murine tumours called teratocarcinomas. EC cells are pluripotent and resemble embryonic cells at a time before X-inactivation has taken place. I have attempted to study X-chromosome activity in EC cells in two ways. (i) In the main project I attempted to create female EC cells which carried human or Chinese hamster X-chromosomes by cell hybridization techniques. An inability to recover these and other hybrids involving EC cells provoked an investigation into the formation and fate of heterokaryons formed between EC cells and other cell types. The results of this study suggest that heterokaryons formed between EC cells and some differentiated cell types do not proliferate due to an incompatibility between the pluripotent and differentiated states. (ii) In addition, I attempted to determine whether a female EC cell line (P10) had two active X-chromosomes by measuring and comparing the specific activities of three X-coded and two autosomally coded enzymes in it and in EC cells which had a single functional X-chromosome. X-coded enzyme levels in P10 and subclones of P10 were not twice as high as in EC cells which expressed a single X-chromosome. Furthermore, X-coded enzyme levels did not drop by half upon the differentiation of P10 and subclones of P10. Concurrent with these experiments others showed, by independent criteria, that P10 and some subclones of P10 had two active X-chromosomes,

and that X-inactivation did occur upon the differentiation of these cells. In view of these facts, it must be concluded that X-coded enzyme levels do not reflect the number of active X-chromosomes in P10 and its derivatives.

RÉSUMÉ

Les carcinomes embryonaires (CE) sont les cellules souches des tumeurs de souris appelées teratocarcinomes. Les cellules CE sont pluripotentielles et ressemblent aux cellules embryonaires avant que l'inactivation du chromosome-X se soit produite. J'ai tenté d'examiner l'activité des chromosomes-X des cellules CE de deux façons. (i) Dans la principale partie du projet, j'ai tenté de créer par les techniques d'hybridation des cellules CE femelles portant des chromosomes-X d'humain ou d'hamster chinois. Vu l'impossibilité d'obtenir ceux-ci ainsi que d'autres hybrides impliquant les cellules CE, une investigation de la formation et du destin des hétérocaryons formés à partir des cellules CE et d'autres types cellulaires a été poursuivie. Les résultats de cette étude suggèrent que les hétérocaryons formés à partir des cellules CE et certains types cellulaires différenciés ne se prolifèrent pas à cause de l'incompatabilité entre la pluripotentialité et l'état différencié. (ii) J'ai aussi essayé de determiner si une lignée cellulaire femelle CE (P10) avait deux chromosome-X actifs. Les activités spécifiques de trois enzymes codés par le chromosome-X et de deux codés par des autosomes dans P10 et dans les cellules CE qui n'ont qu'un chromosome-X fonctionel ont été mesurées et comparées. L'activité des enzymes codés par le chromosome-X dans la P10 et dans les sous-clones de P10 n'est pas deux fois plus élevé que dans les cellules CE qui n'ont qu'un

seul chromosome-X. Elle n'est pas non plus diminuée de moitié lors de la différenciation de P10 et des sous-clones de P10. En même temps, et par des critères différent, d'autres chercheurs ont démontré que le P10 et les sous-clones de P10 avaient deux chromosomes-X actifs et que l'inactivation du chromosome-X se produisait lors de la différenciation de ces cellules. On peut donc conclure de ces résultats que les niveaux d'enzymes codés par le chromosome-X ne reflètent pas le nombre de chromosomes-X actifs dans le P10 ou dans ses dérivés.

CHAPTER 1

INTRODUCTION

CONTENTS:

X-CHROMOSOME ACTIVITY AND EMBRYONAL CARCINOMA CELLS

INTRODUCTION

The study of embryonic development presents a challenge to many biologists today. The mechanisms which prevail in the developing embryo are only slowly being brought to light. Of central importance to an understanding of the process of embryogenesis is the elucidation of the role and manner of differential gene expression.

It seems clear that different genes must function at various times throughout the course of embryogenesis. As development proceeds, cells destined to form particular tissue types must activate genes whose products are necessary for differentiation. By the same token, some genes must be repressed once their products have acted to the appropriate extent. Transcription is the first of many levels at which gene expression may be regulated. Our understanding of transcriptional control in eukaryotes remains vague.

X-chromosome inactivation

Models of gene regulation are studied in the hope of defining control mechanisms which may have a broad applicability. One such model is the inactive mammalian X-chromosome.

Female mammals carry two X-chromosomes in all somatic

cells while males have an X and a Y-chromosome. Early in the embryogenesis of the female, one of the two X-chromosomes in every cell is inactivated at random. This inactive X is inherited through subsequent mitosis. Thus, each somatic cell of the female contains a transcriptionally inert X-chromosome.

X-inactivation is an interesting developmental event in itself, and, an understanding of the mechanism of X-inactivation may shed light on the subject of eukaryotic gene control in general.

In 1961 Lyon (1) and Russell (2) first proposed that one X-chromosome in every female somatic cell is inactivated at random at some point in embryogenesis. This hypothesis was based on observations on coat colour variegation in female mice heterozygous for X-linked coat colour alleles. A number of other observations have given strong support to the inactive X hypothesis. Some of this evidence is presented below.

(i) The heterochromatic X-chromosome

A heterochromatic X, which replicates late in S phase, has been observed in the somatic cells of all female mammals so far examined (3). In some cases the late replicating X can be identified as that X whose gene products are not present in a clonal cell culture (3). This is in agreement with the accepted notion that heterochromatin is transcriptionally inactive (4).

(ii) XO mice and humans

Mice which are karyotypically XO are normal fertile females (3). XO humans are nearly normal females as well. Thus, a single active X-chromosome is sufficient for proper development in mice and humans.

(iii) Coat colour variegation

Female mice heterozygous for X-linked coat colour alleles display discrete patches of one or the other hair colour. Each patch is the result of the clonal proliferation of melanocytes (5) which express only one coat colour allele.

(iv) Absence of gene product

The absence of X-linked gene products provides the most convincing evidence for X-inactivation. Cloned cultures of fibroblasts from women heterozygous for the A and B electrophoretic variants of the X-linked enzyme glucose-6-phosphate dehydrogenase (G6PD) display a single form of the enzyme (6). This restriction of allelic expression has been demonstrated for other X-linked genes in humans and other mammals (3).

(v) Specific activities of X-linked enzymes

Epstein et al (7) showed that early female mouse embryos had approximately twice the specific activities of X-linked enzymes as did male mice. This difference can not be demonstrated in older mouse embryos (8). Assuming that

X-linked enzyme levels are proportional to the number of active gene copies which code for them, these data suggest that both X-chromosomes are active in the very early embryo and one of them subsequently becomes inactivated.

Taken together these findings provide strong evidence for X-inactivation in the mammalian female.

X-chromosome inactivation ensures that female (XX) and male (XY) mammals express the same number of X-linked gene copies. This dosage compensation presumably arose concurrently with the evolution of the heteromorphic sex chromosome pair in order to prevent differential selection between the sexes (9). Once established, dosage compensation by X-inactivation must have provided a selective force for the retention of X-linked genes on the X-chromosome (10). This is suggested by the finding that numerous homologous genes have been shown to be X-linked in a variety of mammals (11,12). Conversely, no gene which has been shown to be X-linked in one mammalian species has ever been shown to be autosomally inherited in another (11). Furthermore, the X-chromosome comprises about 5% of the genome and is about the same size in the great majority of mammalian species studied (10). These observations suggest that X-inactivation as a means of dosage compensation arose about 100 million years ago (11)

Interest in X-inactivation has centered on the chronology of the event in embryogenesis and the molecular mechanisms by which it is accomplished. Relative to the above a number of points must be considered. (i) Both X-chromosomes

are initially active (7). The process is therefore one of inactivation, and not activation as proposed by some (13). (ii) Inactivation of the paternally or maternally inherited X-chromosome is equally likely in the embryo proper as determined by cytogenetic and biochemical marker studies (3). (iii) The paternally inherited X is preferentially inactivated in the extraembryonic membranes of the rat (14) and mouse (15,16,17). (iv) Inactivation commences around the time of implantation in the mouse embryo (3). (v) The process may precede the commitment of each embryonic cell to a differentiative path (18). (vi) Inactivation is generally irreversible. The same X is maintained inactive from mitosis to mitosis (6,19). The derepression of a small region of an inactive human X-chromosome in a mouse/human hybrid cell line has been reported (20) and recently confirmed (21). (vii) There is a locus on the inactive human X-chromosome which is not repressed (23,24). (viii) The number of active X-chromosomes may be determined by the number of maternal haploid autosome sets (22). Any model of X-inactivation must take these observations into account.

Nothing is known about the mechanism by which X-inactivation is initiated and maintained. The most popular models involve either the cooperative binding of regulatory proteins to an effector locus (13,22,25), or, the specific modification of DNA via base methylation (26,27). They have not been experimentally tested.

Two factors pose major problems to the study of X-inactivation. Firstly, the embryo consists of a small number of cells when X-inactivation occurs and so provides little material for study. Secondly, development takes place in utero and so is only observable through surgery. There is a definite need to obtain a system which surmounts these technical difficulties. Embryonal carcinoma cells of the mouse may provide such a system.

Embryonal carcinoma cells

Murine embryonal carcinoma (EC) cells are the stem cells of tumours known as teratocarcinomas. These tumours can arise spontaneously from germ cells in the gonads of certain strains of mice (28,29), or they can be induced by the transplantation of an early mouse embryo to an extra-uterine site, such as beneath the testis capsule (30,31,32,33). The subsequent disorganized growth of the embryo forms the tumour. Teratocarcinomas can be induced by other means as well, such as the introduction of zinc metal strips into the testis (34). Independent of their origin, teratocarcinomas are composed of a wide variety of differentiated tissue types in addition to EC (34).

EC cells are very similar to cells of the early embryo. This is apparent in their ability to differentiate along numerous pathways. Individual EC cells have been shown to be pluripotent (35). When injected into early mouse embryos they will participate in the development of a

viable mouse (36). They can thus be subjected to the same developmental constraints as normal embryonic cells.

What renders EC cells of considerable value is their ability to be grown in tissue culture (37). Under the appropriate in vitro conditions they will form numerous differentiated cell types (38,39). One may observe endoderm, epithelium, muscle, nerve and other differentiated tissues. EC cells thus provide a limitless source of experimental material with which differentiation can be studied. They can also be subjected to a variety of manipulations available for cells in culture. Furthermore, EC cells are functionally equivalent to embryonic cells at a time before X-inactivation has taken place (40). Thus, the use of EC cells avoids the technical difficulties encountered when working with early embryos. This makes them an ideal system in which to attempt the study of X-inactivation.

There are to date only two published papers involving the study of X-chromosome activity in EC cells. The first, by McBurney and Adamson (1976) (40), characterized two karyotypically XX and three XO embryo-derived EC cell lines for the specific activities of four X-coded enzymes. Levels were similar in XX and XO cells suggesting that all lines had only one functioning X-chromosome. This was supported by the observation that the frequency of recovery of recessive X-linked mutations was comparable in XX and XO cells. Only one of the two XX cell lines had a demonstrably late replicating X-chromosome. This may suggest that X-inactivation

precedes entry into a late replicating state. Different EC lines could be functionally equivalent to developmental stages prior to and after entry into late X-chromosome replication.

Martin et al (1978) (41) measured the specific activities of three X-coded enzymes in an XX EC cell line derived from an ovarian teratocarcinoma and in three XO EC cell lines of testicular origin. The levels were twice as high in the XX as compared to the XO cell lines. When these cells were made to differentiate the specific activities in the female line dropped to levels similar to those of the XO. The conclusion drawn was that X-inactivation had taken place during differentiation. There was no report of the identification of a late replicating X-chromosome in the differentiated cultures.

The continued study of X-chromosome activity in EC cells is warranted. It is of interest to determine whether female EC cells carrying two active X-chromosomes can be isolated from embryo-derived teratocarcinomas as well as parthenogenetically derived ones. The production of EC cells with electrophoretically separable X-coded enzyme forms would be most useful. The practical applications of such cells are discussed in a later section.

Genetic manipulation via cell fusion

Experimentally induced cell fusion has proven to be a powerful tool upon which the field of somatic cell genetics

has been established. Cell hybrids have been useful in such areas as the characterization of biochemical lesions through complementation, the search for regulators of differentiation through the fusion of cells of differing developmental status, and the mapping of the human genome in man/mouse hybrids (42).

Cell fusion is most commonly effected through the use of inactivated Sendai virus or polyethylene glycol (PEG). How plasma membranes fuse is not well understood, although various parameters necessary for it to occur have been defined (42).

When a mixture of two cell types is treated with a fusion inducing agent, fusion may occur between cells of the same or of different types. These fusion products are termed homokaryons and heterokaryons, respectively. Their survival depends on a number of factors. Those formed by the fusion of two cells only are the most likely to proliferate (42). Of these, most do not survive the first few mitoses as a result of complications in spindle arrangement and chromosome movement, probably due to the presence of extra centriole pairs (42). The daughter cells of heterokaryons are true hybrids, composites of the cytoplasms and chromosomes of the two parental cells.

One notable characteristic of hybrids is the tendency to lose chromosomes. This process is termed segregation (42). The chromosome complement becomes progressively more stable with continued proliferation. In the case of mouse/human hybrids there is a preferential loss of human chromosomes (43).

Virtually all of the human chromosomes can be lost.

Cell hybridization techniques can be used to transfer small numbers of chromosomes from one cell type to another. Microcells and mini segregants are two types of fusable cell fragments which are used for this purpose. Microcells are derived from cells that contain micronuclei. Micronuclei are small nuclear structures (fig. 1) which have less than the diploid number of chromosomes. Some rodent cell lines form micronuclei in response to prolonged mitotic arrest (42). When micronucleated cells are subjected to high-speed centrifugation in Ficoll density gradients containing cytochalasin-B, the micronuclei are forced from the cell in a thin shell of cytoplasm and plasma membrane to produce microcells (44, 45). The DNA content of microcells can be as small as that of a single chromosome (45). Microcells provide a vehicle by which a single chromosome may be transferred to another cell through membrane fusion.

Mini segregants are derived from HeLa cells by another method (46,47). HeLa cells undergo aberrant cytokinesis after exposure to mitotic inhibitors and low temperature. The cells constrict in numerous planes to form multi-lobed structures. Because some of these structures resemble a bunch of grapes (fig. 2), each abnormally cleaved cell is called a BOG, for short. Isolated lobes called mini segregants can be obtained by the passage of BOG's through a hypodermic needle. Like microcells, mini segregants contain a variable number of chromosomes and can be used in cell

Figure 1: Photograph of an intact 3T3 nucleus beside one which has undergone micronucleation due to prolonged mitotic arrest.

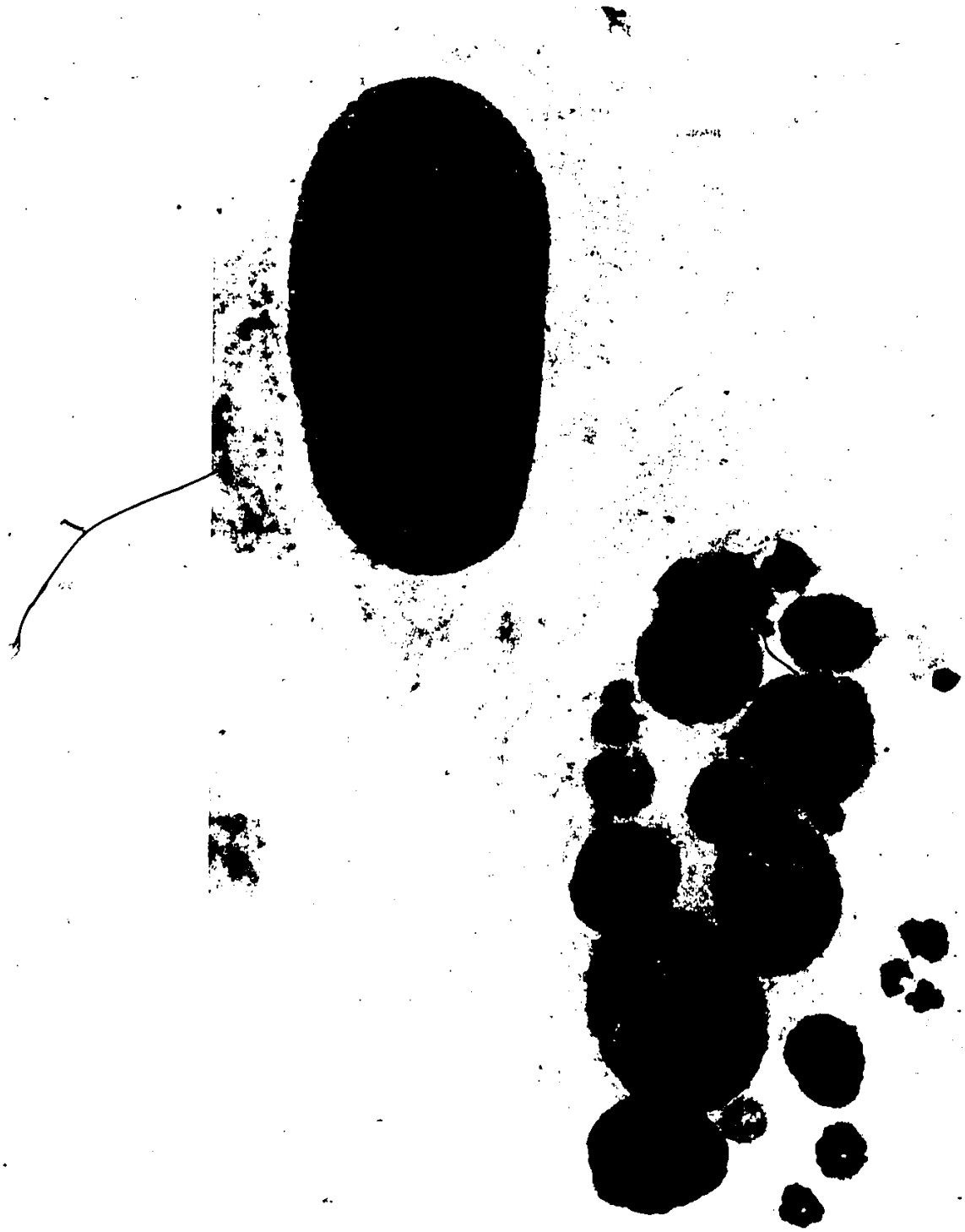
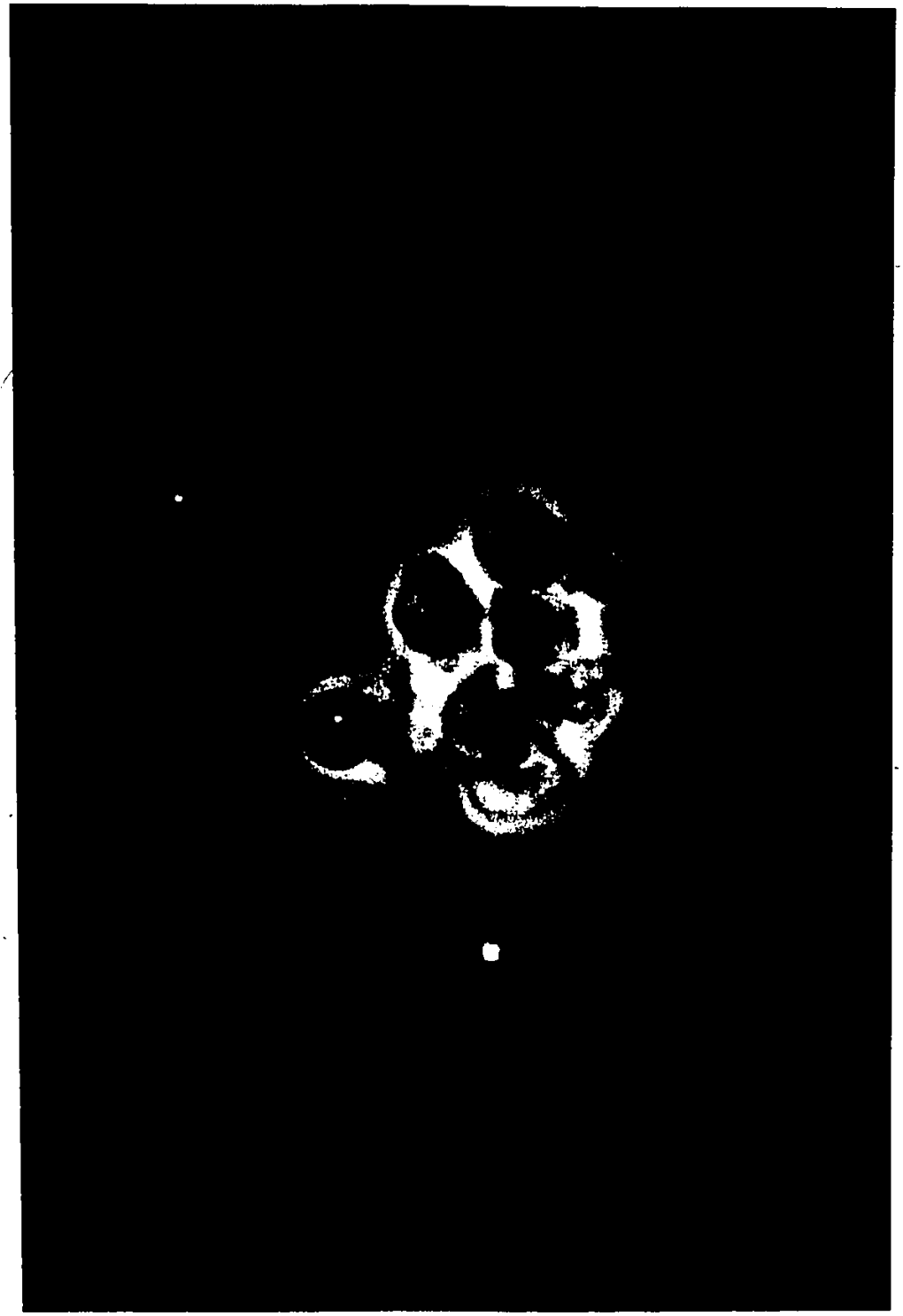


Figure 2: HeLa BOG produced by aberrant cytokinesis after prolonged mitotic arrest.



fusions (48).

Methods are available for the selection of hybrid cells over unfused cells and homokaryons. One way is to fuse cells which have different genetic markers or biochemical deficiencies and select under conditions in which only the hybrids can grow. For example, cells must possess both of the enzymes hypoxanthine-guanine phosphoribosyl transferase (HGPRT) and thymidine kinase (TK) in order to survive in the selective medium known as HAT (49, 50,51). Cells which are HGPRT or TK deficient can be obtained by selection for resistance to toxic nucleotide analogues (49). The fusion of $HGPRT^-$ cells to TK^- cells will produce hybrids which will grow in HAT (due to complementation) whereas the parental cells fail to survive. The gene for HGPRT is X-linked in all mammalian species in which it has been mapped (52,53). Thus, hybrids which retain the X-chromosome of the $HGPRT^+$ parent are selected in HAT.

The proposed projects

(i) Production of embryonal carcinoma cells carrying foreign X-chromosomes

The aim of the project I initially undertook was to introduce an X-chromosome of human or Chinese hamster origin into the female $HGPRT^-$ EC cell line C145A12 (54). Such a hybrid would be useful for the study of X-chromosome activity because mouse and non-mouse X-coded enzymes can be separated electrophoretically. The most valuable enzyme in this

context would be glucose-6-phosphate dehydrogenase (G6PD). G6PD is a dimer. When two electrophoretically separable forms of G6PD are synthesized in the same cell, subunits of each type combine to form heterodimers. Heterodimers are observed as an electrophoretic band of G6PD activity which migrates between the two homodimers (55). Band intensities are proportional to the cytoplasmic concentration of G6PD. Cytoplasmic concentration of the enzyme is in turn proportional to the number of active genes coding for it. This enzyme thus provides a means of determining the ratio of the number of active X-chromosomes carrying the gene for one form of G6PD as opposed to another.

X-chromosome activity in this hybrid could also be monitored by means of the cytogenetic and biochemical techniques discussed earlier.

The hybrid could be used to answer two questions. (i) The number of active X-chromosomes in C145A12 could be determined by the G6PD electrophoretic pattern of the proposed hybrids. (ii) If the hybrids could be induced to differentiate they could be tested for the occurrence of X-inactivation. If it did take place the randomness of inactivation and its timing with respect to other differentiation events could be determined, also through G6PD electrophoretic analysis. In mechanistic and evolutionary terms it would be of considerable interest to determine whether a non-mouse X-chromosome could be inactivated in a mouse cell.

The transfer of a foreign X-chromosome to C145A12 was attempted in three ways. C145A12 cells were fused to (i) HeLa mini segregants and (ii) microcells of Chinese hamster ovary cells. (iii) Fusions between whole HeLa and C145A12 cells were also performed with the intention of obtaining hybrids which had lost all human chromosomes but the X. In addition, whole CHO cells were fused to C145A12 cells. These last two experiments provided a means of assessing the efficiency of hybrid formation between EC cells and mini segregants or microcells by establishing the frequencies of whole cell hybrid recovery.

In spite of considerable effort, no hybrids were recovered from any of these experiments. We had noted earlier that we were able to recover hybrids between EC cells and some cell types, but not with others. This observation, plus the lack of success in the above experiments, suggested that there was a block in hybrid formation between EC cells and certain cell types. We felt that such a block might also be responsible for an inability to recover hybrids between EC cells and microcells and mini segregants. I therefore undertook to determine the site at which viable hybrid formation was blocked and, if possible, use this information to bypass the block and produce C145A12 cells carrying foreign X-chromosomes. This work expanded to form the bulk of this thesis and is presented in chapter two.

(ii) X-coded enzyme levels in embryonal carcinoma cells

Male and female EC cell lines have recently been

established in culture in our laboratory from embryo-derived teratocarcinomas of C3H mice. We wished to determine (i) whether the female EC cell lines had one or two active X-chromosomes, and if so, (ii) whether X-inactivation occurred upon differentiation.

One manner in which these problems can be addressed is through the measure of specific activities of enzymes coded for by X-linked genes. There is evidence that the specific activity of X-coded enzymes is directly proportional to the number of active X-chromosomes (7,8,56,57,58). Female EC cells with two active X-chromosomes should have twice the level of X-coded enzymes as male EC cells and EC cells with one active X. The specific activities of three X-linked enzymes were determined for a number of EC lines. This work was part of a collaborative effort; independent direct assessments of X-chromosome activity were simultaneously performed. The results of this investigation are presented in chapter three.

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CHAPTER 2

FORMATION AND FATE OF HETEROKARYONS INVOLVING EMBRYONAL CARCINOMA CELLS

CONTENTS:

ABSTRACT

We have shown that murine embryonal carcinoma (EC) cells will readily fuse to non-EC cells following treatment with polyethylene glycol (PEG). Heterokaryons were detected by autoradiography and premature chromosome condensation (PCC). Those heterokaryons formed between EC cells and certain of the non-EC cell types did not proliferate to form viable hybrids. In contrast, viable hybrids were isolated following fusion of the differentiated progeny of EC cells and one of these non-EC cells. The evidence suggests that pluripotent and certain differentiated genomes are incompatable in a common cytoplasm.

INTRODUCTION

The techniques of cell hybridization have been used to investigate the nature of intracellular regulators of gene expression (1). Pluripotent murine embryonal carcinoma (EC) cells may be unique for these studies because they are developmentally naive (2). Hybrids between EC cells and a number of differentiated cell types have been isolated. In some cases the hybrids resembled EC cells (3-6) while in others the developmental status of the hybrids was not clear (7-10). Still others displayed characteristics of the differentiated parent (5,11-15). An example of the latter situation is EC/Friend cell hybrids which synthesize hemoglobin like the Friend cell parent (16). The EC-derived globin genes of these hybrids are expressed providing evidence for the presence of positive regulators of globin gene expression (17).

It would be of interest to determine the developmental status of hybrids formed following the fusion of EC cells with cells displaying other differentiated characteristics. We have found, however, that many cell types do not form viable hybrids with EC cells. The evidence presented below indicates that this is because the heterokaryons do not proliferate.

MATERIALS AND METHODS

Cell lines and culture techniques

All cell lines were routinely cultured in plastic Petri dishes in alpha-modified minimal essential medium (18) supplemented with 10% fetal calf serum (FCS) (Flow Laboratories, Mississauga, Ont.) in a 5% CO_2 humidified incubator. Cells were removed from the dishes by a 5 minute treatment with Ca^{2+}-and Mg^{2+}-free phosphate buffered saline (PBS) containing 1 mM ethylene diamine tetraacetic acid (EDTA) and 0.025% trypsin. The cell lines used in this investigation are described in table 1.

Cell fusion and hybrid selection

Cells attached to the bottom of glass scintillation vials were fused by a modification of the procedure of Davidson and Gerald (19). One ml of a sterile aqueous solution of 0.1% (W/V) poly-L-lysine (M.W. 500,000) (Sigma) was placed in a sterile 20 ml glass scintillation vial (Kimble, Skokie, Ill.) for 5 sec. The solution was then removed and the vial washed with 5 ml of sterile PBS. 1.5×10^6 cells of each type to be fused were mixed in a total volume of 2 ml serum-free medium and added to the vial. The vial was centrifuged (700 g) to produce a semi-confluent monolayer of cells. All residual medium was aspirated from the vial and 1 ml of sterile 50% (W/V) polyethylene glycol (PEG) 1000 (Koch-Light Laboratories,

Colnbrook Bucks, England) made up in serum-free medium was added to the vial so as to cover the cell monolayer. This solution was removed after 60 seconds and the cell monolayer was washed twice with 10 ml of serum-free medium. Five ml of medium supplemented with 10% FCS were added to the vial and the cells were incubated for 1 hr at 37°C. The cells were then removed from the bottom of the vials by trypsinization and 1.5×10^6 cells were plated at 10^5 cells/ml into 100 mm plastic Petri dishes. Selective agents were added the next day. The following selective media were used: HAT (20) (10 ug/ml hypoxanthine, 0.1 ug/ml methotrexate, 10 ug/ml thymidine); HAT plus 1 uM ouabain.

Autoradiography

The cells to be labeled were cultured for 24 hrs in medium containing 1 uCi/ml ^{3}H-thymidine (dT) (Amersham/ Searle). Labeled cells were washed, fused to unlabeled cells, and plated onto sterile glass coverslips at 2.5×10^5 cells/ml in medium containing 10 ug/ml unlabeled dT. HAT and ouabain were added the next day if prolonged culture was carried out. Cells adhering to coverslips were fixed with 3:1 methanol: acetic acid and the coverslip mounted cell-side up on microscope slides. The slides were dipped in a 50% (V/V) aqueous solution of NTB2 nuclear track emulsion (Eastman, Rochester, N.Y.) containing 1% glycerol and exposed for one week in the dark at 4°C. The autoradiographs were developed in undiluted Kodak D19 developer for 3.5 min. Slides were

then stained in 2% Giemsa (pH 6.8) for 10 to 20 min.

Premature chromosome condensation

The procedure for the induction of premature chromosome condensation was based on that of Johnson and Rao (21). HeLa cells were collected in metaphase by culturing cells for 24 hrs in the presence of 0.06 ug/ml of Colcemid (Cal Biochem, La Jolla, Cal.). Metaphase HeLa cells were fused to EC or 3T3 cells by the procedure outlined above. The cells were incubated at 37°C for 30 min after PEG treatment. Chromosome spreads were then prepared and stained in 2% Giemsa (pH 6.8) for 5 min.

Chromosomes, GPI and G6PD analysis

Chromosome spreads were prepared by the air drying method and stained with Giemsa. Ten or twenty spreads from each clone were counted. Electrophoresis of glucose phosphate isomerase (GPI) and glucose-6-phosphate dehydrogenase (G6PD) was carried out on starch gels (16) and cellulose acetate gels (22), respectively.

Isolation of HGPRT$^-$ mutants and TK$^-$ segregants

HGPRT deficient 3T3 cells were selected in 6-thioguanine (5 ug/ml). Colonies arose at a frequency of about 10^{-6}. 3T3H1 is a clone derived from this selection.

TK deficient segregants were obtained by growing hybrids in 8-azaserine (0.02 mM) (Cal Biochem, La Jolla, Cal.) plus

hypoxanthine (10 ug/ml) for two weeks followed by selection in BrdU (30 ug/ml). TK^- segregants were routinely cultured in the presence of 8-azaserine plus hypoxanthine to select for cells which are $HGPRT^+$ (23).

Mini segregant and microcell production

HeLa mini segregants were prepared according to the method of Johnson et al (24). Microcells were prepared by exposing cells to Colcemid for 48 hrs (1) and then centrifuging them in Ficoll density gradients (25,14). Microcells and mini segregants which passed through a 5.0 u Uni-Pore filter (Bio-Rad, Mississauga, Ont.) were used in order to enrich for those carrying small numbers of chromosomes.

Differentiation of EC cells in vitro

P19A2 cells were induced to differentiate by culturing the cells in the presence of 10^{-7} M retinoic acid (26) for one to two weeks. The medium was changed every second day. This procedure yielded a differentiated cell type, probably primitive extra-embryonic endoderm (E. Jones-Villeneuve, personal communication).

TABLE 1

DESCRIPTION OF CELL LINES

Cell Name	Cell Type	Selective Markers Used in this Study	Source or Reference
C145A12	murine EC	$HGPRT^-$	14
P19A2	murine EC	$HGPRT^-$	M. McBurney
3T3	murine (swiss) fibroblasts	TK^-	I. Craig
3T3H1	murine (swiss) fibroblasts	$HGPRT^-$, TK^-	present paper
HeLa	human epithelial carcinoma	sensitive to 1uM ouabain	M. Rauth
IMR90	human embryonic fibroblasts	sensitive to 1 uM ouabain	W. Nichols
FdU8B	chinese hamster ovary cells	TK^-	L. Siminovitch
EOHM	chinese hamster ovary cells	$HGPRT^-$	R. Warton
STO	murine fibroblasts	$HGPRT^-$	A. Bernstein

Abreviations: $HGPRT^-$ = deficient in hypoxanthine-guanine phosphoribosyl transferase; TK^- = deficient in thymidine kinase.

RESULTS

We have been unable to recover cell hybrids following the fusion of four independently isolated EC cell lines to certain non-EC cell types such as murine primary fibroblasts and myeloma cells, human fibroblasts and lymphocytes, HeLa cells and CHO cells. In the last two examples the frequency of hybrid recovery was less than 10^{-7} hybrids per cell plated.

On the other hand 3T3 fibroblasts gave rise to viable hybrids with EC cells at a frequency of about 4×10^{-3}. We have also recovered hybrids of EC and Friend cells at lower frequencies (10^{-5} to 10^{-6}) (16); however, our general observation has been that EC cells form viable hybrids with very low frequency following their fusion to many non-EC cell types.

Detection of heterokaryons

Our inability to recover hybrids could be because (i) plasma membrane fusion does not take place, or, (ii) heterokaryons formed following membrane fusion do not proliferate. To test these alternatives we have looked for heterokaryons following polyethylene glycol (PEG) treatment of mixed cultures.

We used autoradiography in the first test for the presence of heterokaryons. The DNA of cells of the EC line C145A12 was labeled by growing the cells in the presence of ^{3}H-dT. These cells were fused to unlabeled human embryonic

fibroblasts (IMR90) or to mouse 3T3 fibroblasts. The fusion mixtures were plated onto coverslips and these were prepared for autoradiography. Heterokaryons were identified by the presence of labeled and unlabeled nuclei in the same cytoplasm. C145A12/IMR90 heterokaryons (fig. 1) were detected at a similar frequency to C145A12/3T3 heterokaryons (table 2). Dikaryons, which are the heterokaryons most likely to form hybrids (1), made up 1.2% and 1.0% of the C145A12/IMR90 and C145A12/3T3 post fusion mixtures, respectively. This experiment demonstrates that EC cells are quite capable of forming heterokaryons with IMR90 fibroblasts.

Neither EC nor HeLa cells spread well on glass coverslips. This would make the autoradiographic detection of EC/HeLa heterokaryons difficult. However, virtually 100% of a HeLa cell culture can be blocked at metaphase by a 24 hr exposure to Colcemid. The fusion of an interphase cell with a cell in mitosis results in the premature condensation of the interphase chromosomes (1). Prematurely condensed (PC) chromosomes of cells in G_1, S and G_2 cell cycle phases are distinguishable from each other and from metaphase chromosomes. We, therefore, used the induction of premature chromosome condensation (PCC) as a means of assessing the extent of fusion between HeLa and EC cells.

HeLa cells blocked at metaphase were fused to C145A12 cells or to 3T3 fibroblasts and chromosome spreads were subsequently prepared. The presence of PC chromosomes associated with a HeLa metaphase chromosome spread was taken

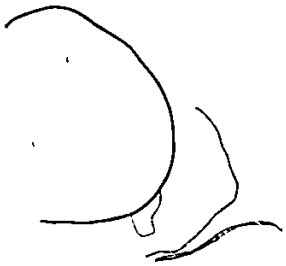


Figure 1: Example of a heterokaryon detected by autoradiography. Photograph shows a heterokaryon formed by the fusion of a ^{3}H-dT labeled C145A12 cell to an unlabeled IMR90 cell. Photographic grains are found at high density over the C145A12 nucleus.

TABLE 2

HETEROKARYON AND HYBRID FORMATION[1]

Fusion Mixture	Percent Heterokaryons[2]	Hybrid Colonies Per 10^6 Cells Plated
C145A12 x IMR90	13.9	0
C145A12 x 3T3	4.8	820
C145A12 x HeLa	3.8	0
HeLa x 3T3	7.2	400

1 Heterokaryon formation was scored by PCC or autoradiography (see text). Hybrids were selected from fusions involving unsynchronized HeLa cells and unlabeled C145A12 cells.

2 Percent heterokaryons as determined by autoradiographs was defined as the number of labeled (C145A12) nuclei in heterokaryons divided by the total number of labeled nuclei, times one hundred. 500 cells were scored. Percent heterokaryons as determined by PCC was defined as the number of HeLa chromosome spreads containing PC chromosomes divided by the total number of HeLa chromosome spreads, times one hundred. 500 HeLa chromosome spreads were scored.

as an indication that fusion had occurred.

The extent of fusion in the 3T3/HeLa and C145A12/HeLa mixtures was comparable (table 2). Figure 2 shows PC chromosomes resulting from the fusion of mitotically arrested HeLa cells to C145A12 cells in G_1, S and G_2. The number of PC chromosomes can be counted in the photographs of G_1 and G_2 PCC and is 40 in both cases (C145A12 cells have 40 chromosomes). Furthermore, all the G_2 PC chromosomes appear to be telocentric, a characteristic of all normal mouse chromosomes. The extent of cell fusion was a function of the duration of exposure of the mixed cell monolayer to PEG (fig. 3). No PC chromosomes were detected in the absence of PEG treatment. These results indicate that fusion does take place between EC cells and mitotically arrested HeLa cells.

For each experiment designed to detect heterokaryon formation a parallel experiment was performed to determine the frequency of hybrid formation. These experiments made use of unsynchronized HeLa cells and unlabeled C145A12 cells. The results are presented in table 2. Although heterokaryons were present at high frequency in all fusion mixtures, no hybrids were recovered from fusions of C145A12/HeLa or C145A12/IMR90. In these cases the heterokaryons apparently do not proliferate. On the other hand, cell hybrids were recovered at high frequency from C145A12/3T3 and 3T3/HeLa fusion mixtures.

Figure 2: Prematurely condensed EC chromosomes following fusion of interphase EC cells to mitotically arrested HeLa cells. The HeLa chromosomes are short and thick. a) G_1 C145A12 PC chromosomes are long and thin and composed of a single chromatid. b) S phase C145A12 PC chromosomes have a beaded or pulverized appearance. c) G_2 C145A12 PC chromosomes are long and thin telocentrics composed of two chromatids.

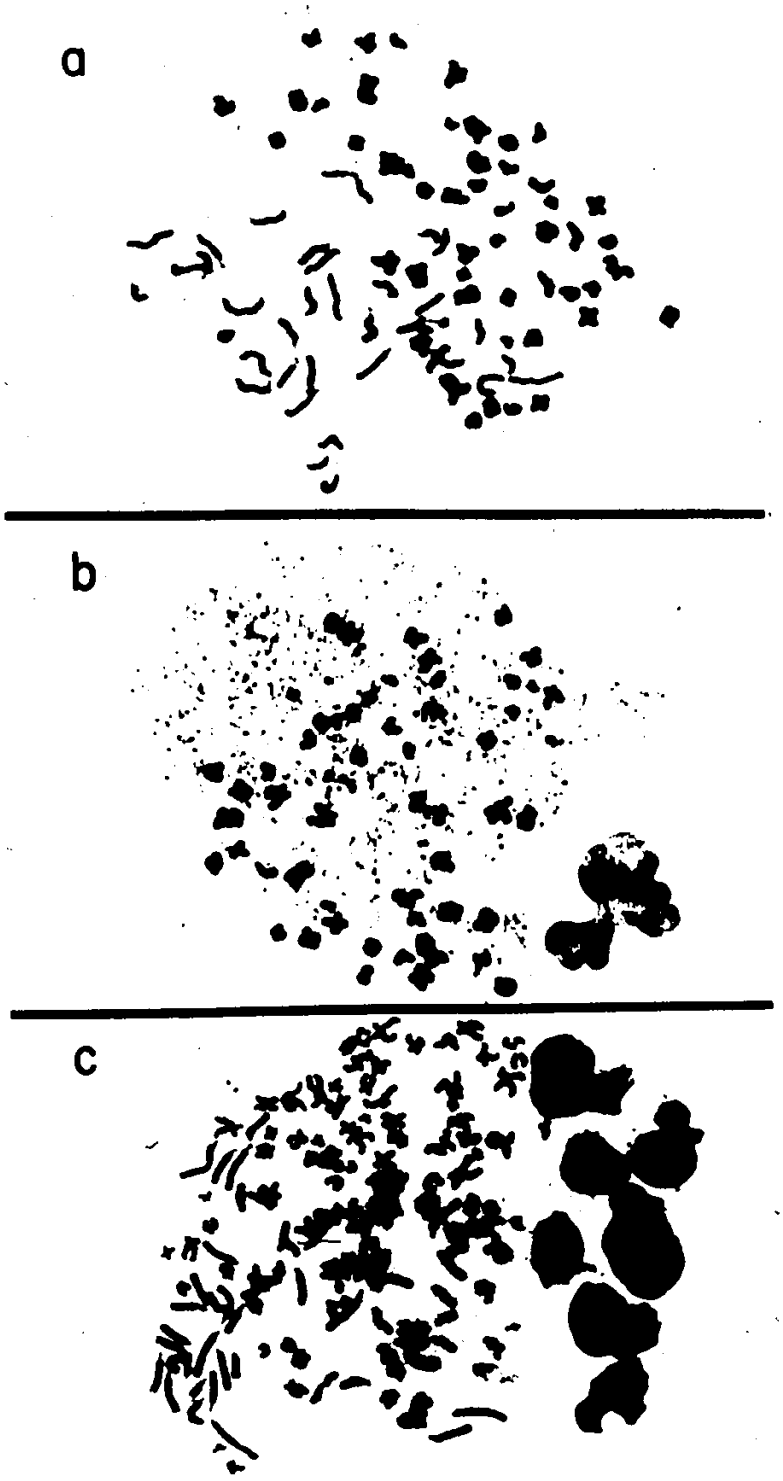
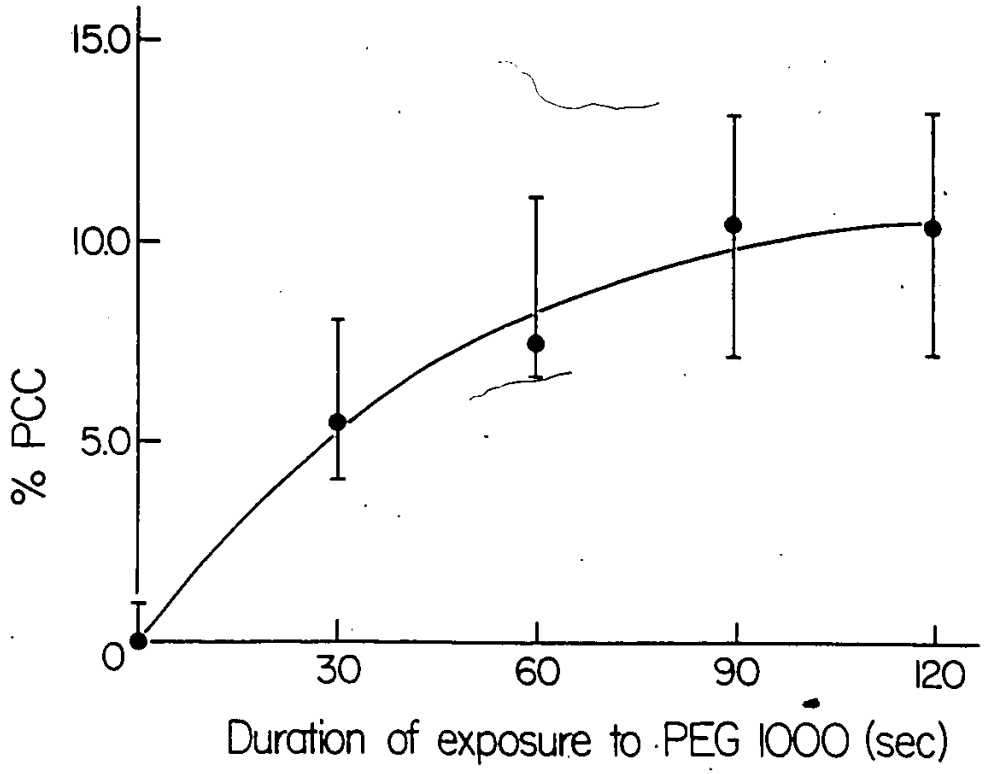

a
b
c

Figure 3: Effect of the length of PEG exposure on the frequency of PCC induction. The percentage of prematurely condensed EC chromosomes at each exposure was determined from 500 HeLa chromosome spreads. Bars indicate 95% confidence intervals.


15.0
10.0
5.0
0
% PCC
30
60
90
120
Duration of exposure to PEG 1000 (sec)

Heterokaryon survival

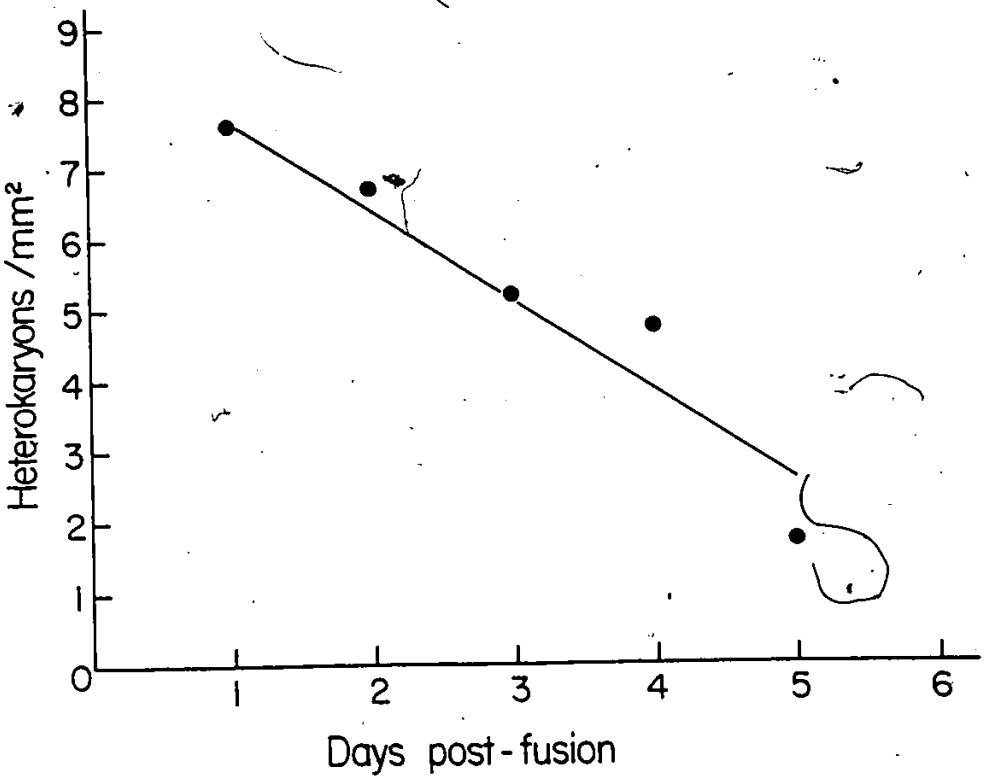
EC cells have high levels of deoxyribonuclease activity (27). One possible reason for the inability of C145A12/IMR90 heterokaryons to form viable hybrids is that one or both nuclei are rapidly destroyed by hydrolytic enzymes such as DNase I. To examine the long term fate of heterokaryons we performed the following experiment.

C145A12 cells prelabeled with ^{3}H-dT were fused to IMR90 fibroblasts and equal aliquots of the fusion mixture were plated onto a number of glass coverslips in medium containing HAT and ouabain. At daily intervals two coverslips were harvested, prepared for autoradiography and analyzed for the number of heterokaryons present per unit surface area (fig. 4).

Heterokaryon density showed a progressive decrease over the five day period with intact heterokaryons still present on the fifth day following fusion. These results are not consistent with the idea that there is a rapid self-destruction of the heterokaryons. Indeed, the survival of some heterokaryons for several days in HAT and ouabain suggests that both genomes in the heterokaryon are transcriptionally active.

Rechsteiner and Hill (28) have shown by autoradiography that mouse and human chromatin are not intermingled within the nuclei of mouse/human hybrids. Prelabeling one of the parental cell types with ^{3}H-dT gives rise to sectored nuclei within the hybrid cells; exposed grains are found over only about half the hybrid genome. We have observed cells with sectored nuclei in autoradiographs of C145A12/IMR90 fusion

Figure 4: Persistence of heterokaryons with time. C145A12 cells labeled with ^{3}H-dT were fused to unlabeled IMR90 cells and replicate cultures were plated onto glass coverslips. At daily intervals coverslips were fixed, prepared for autoradiography, and subsequently scored for the number of heterokaryons per unit surface area.


Heterokaryons /mm²
0
1
2
3
4
5
6
7
8
9
1
2
3
4
5
6
Days post-fusion

mixtures (fig.5). This observation suggests that some C145A12/IMR90 heterokaryons may undergo cell division. We have only observed pairs of cells with sectored nuclei, even at five days post-fusion. Furthermore, many sectored nuclei display marked morphological abnormalities. Both of these observations suggest that after C145A12/IMR90 heterokaryons pass through one mitosis, their daughter cells cease to proliferate (29).

Microcell fusions

Our inability to recover EC/non-EC hybrids might be the result of the action of a small number of non-EC genes. We wished to determine whether hybrids could be obtained by fusing EC cells to microcells or mini segregants containing small numbers of non-EC chromosomes.

EC cells were fused to HeLa mini segregants and to microcells of CHO, 3T3 and 3T3H1/HeLa C5TK in attempts to transfer foreign X-chromosomes into EC cells. Non-EC X-chromosomes were successfully transferred to EOHM and to STO fibroblasts, but no viable fusion products were isolated when an EC cell was the recipient whole cell.

Tri-hybrid production

3T3 fibroblasts readily form hybrids with either EC cells or HeLa cells. We wished to determine if EC and HeLa genomes could be induced to co-exist in a cell by prior fusion of either to 3T3 fibroblasts.

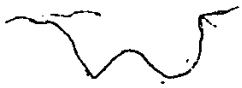


Figure 5: Hybrid cell pair displaying sectored nuclei. These cells were observed on coverslips prepared for autoradiography two days after the fusion of ^{3}H-dT labeled C145A12 cells to unlabeled IMR90 fibroblasts.

3T3H1 cells ($HGPRT^-$, TK^-) were fused to HeLa cells and hybrids selected in HAT plus ouabain. Colonies arose at a frequency of about 10^{-3}. A TK^- segregant of these hybrids, 3T3H1/HeLa C5TK, was isolated and fused to the EC cell lines C145A12 and P19A2. Tri-hybrids were selected in HAT. Colonies arose with a frequency of 2×10^{-4} with C145A12 and 7×10^{-4} with P19A2. Two clones from each experiment were isolated and found to have chromosome numbers (table 3) and GPI and G6PD zymogram patterns (table 3, fig. 6) which confirmed the proposed tri-parental origin.

In the reciprocal experiment tri-hybrids were produced in the following manner. A TK^- segregant of a C145A12/3T3 hybrid, C145A12/3T3 C101TKC1, was fused to HeLa and tri-hybrids selected in HAT plus ouabain. Colonies arose with a frequency of greater than 10^{-5}. Two clones were established and found to have chromosome numbers considerably higher than expected for tri-parental origin (table 3). This situation could have arisen a number of ways. The human form of G6PD could not be detected in these cells, however, human, C145A12 and 3T3 bands of GPI activity, as well as 3T3/HeLa and C145A12/3T3 heterodimeric bands, were present in both clones (table 3, fig. 6). These cells are clearly tri-parental in origin.

Apparently EC and HeLa genomes can co-exist within a viable cell provided the 3T3 genome is also present.

TABLE 3

CHARACTERISTICS OF TRI-HYBRID CELLS

Parental Lines	Chromosome Number Expected[1]	Chromosome Number Observed[2]	Enzyme Forms GPI[3]	Enzyme Forms G6PD[4]
C145A12		40	B	M
P19A2		40	B	M
3T3		65(62-69)	A	M
3T3H1		62(60-65)	A	M
HeLa		55(52-60)	H	H
Hybrids				
3T3H1/HeLa C5TK	117	122(112-132)	A	MH
C145A12/3T3 C101TKC1	105	97(91-109)	AB	M
Tri-hybrids				
C145A12/C5TK S4	162	173(155-186)	AB	MH
C145A12/C5TK S5	162	158(126-172)	AB	MH
P19A2/C5TK S2	162	159(131-176)	AB	MH
P19A2/C5TK S3	162	143(132-155)	AB	MH
C101TKC1/HeLa C1	152	220(200-235)	ABH	M
C101TKC1/HeLa C2	152	224(202-237)	ABH	M

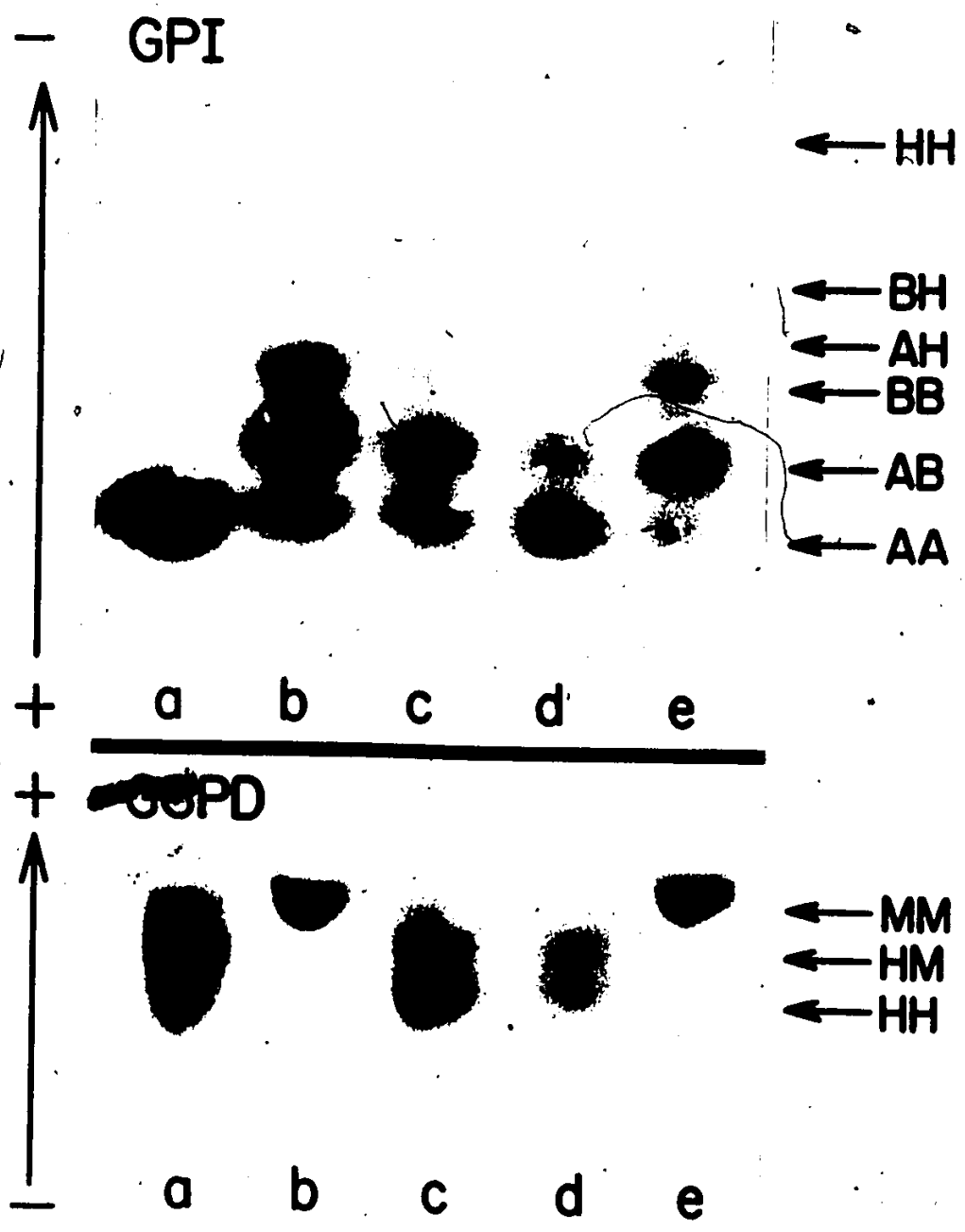
1. Based on sum of modal chromosome number of parental cell lines.

2 Modal chromosome number plus range in brackets.

3 GPI is a dimeric enzyme. A, B, and H in this column indicate that only the murine AA, the murine BB, or the human HH homodimers were present. AB indicates that three bands of activity were present, both the AA and BB murine homodimers and the AB heterodimer. ABH indicates the presence of the three bands of murine GPI activity as well as the human HH homodimer and the AH and BH heterodimers.

4 G6PD is a dimeric enzyme. M and H indicate that just the murine MM or human HH homodimers were present, respectively. MH indicates that three bands of activity were observed, the murine and human homodimers as well as the MH heterodimer.

Figure 6: Electrophoretic analysis of GPI (upper) and G6PD (lower) enzymes present in cell hybrids. The presence of parental bands plus a heterodimeric band(s) of enzyme activity confirms the hybrid nature of these cells. AB designates the heterodimer formed from subunits of the two murine forms of GPI. AH and BH designate the heterodimers formed between subunits of the A or the B murine form of GPI and the human form. Lane a, 3T3H1/HeLa C5TK; b, C145A12/3T3 C101TKC1; c, C145A12/C5TK S5; d, P19A2/C5TK S3; e, C101TKC1/HeLa C1.


—
GPI
HH
BH
AH
BB
AB
AA
+
a b c d e
+
G6PD
MM
HM
HH
—
a b c d e

Hybrid formation with the differentiated progeny of EC cells

EC cells may not give rise to viable hybrids with HeLa or CHO cells due to differences between the pluripotent (EC) and differentiated (HeLa, CHO) states. If this were so, it might be possible to recover hybrids between differentiated progeny of EC cells and CHO or HeLa cells.

Cells of the EC line P19A2 were induced to differentiate into endoderm-like cells by culturing them in the presence of low concentrations of retinoic acid (25) (E. Jones-Villeneuve, personal communication). These differentiated cells and the undifferentiated P19A2 were fused to 3T3 cells, HeLa cells, and the CHO cell line FdU8B. Hybrids were selected in HAT or HAT and ouabain. The frequencies of hybrid recovery are given in table 4.

3T3 cells formed hybrids with both the undifferentiated and differentiated P19A2 (table 5, fig. 7). HeLa cells did not form hybrids in either fusion mixture. While no hybrids were recovered from the fusion of FdU8B to the undifferentiated P19A2, hybrids were recovered at high frequency when FdU8B were fused to the differentiated cells. The GPI zymograms of two clones, P19A2D/FdU8B C1 and C4, established that they were hybrids of mouse and Chinese hamster cells (fig. 7). The large number of telocentric chromosomes in both of the clones (fig. 8, table 5) suggests that they arose by the fusion of one FdU8B cell with one tetraploid or two diploid differentiated P19A2 cells. This is not surprising as tetraploids are common in the differentiated cultures.

TABLE 4

RECOVERY OF HYBRIDS

Fusion Mixture	Hybrid Colonies Per 3×10^6 Cells Plated
Undifferentiated P19A2 x 3T3	108
Undifferentiated P19A2 x HeLa	0
Undifferentiated P19A2 x FdU8B	0
Differentiated P19A2 x 3T3	45
Differentiated P19A2 x HeLa	0
Differentiated P19A2 x FdU8B	30

TABLE 5

CHROMOSOMAL CONSTITUTION OF CELL HYBRIDS

Parental Cell Lines	Number of Telocentrics	Number of Non-telocentrics	Total Chromosome Number
P19A2	40	0	40
FdU8B	0	21	21
3T3	n.d.[2]	n.d.	65(62-69)
Hybrids			
P19A2D/FdU8B C1[1]	70(64-95)	14(8-17)	84(72-109)
P19A2D/FdU8B C4[1]	74(63-95)	13(11-17)	86(74-106)
P19A2/3T3 C2	n.d.	n.d.	106(99-109)
P19A2D/3T3 C1[1]	n.d.	n.d.	105(81-119)

1 These three hybrids were derived from fusions between differentiated P19A2 cultures and FdU8B or 3T3.

2 Not determined

Figure 7: Electrophoretic analysis of GPI enzymes present in hybrids involving differentiated P19A2. CB designates the heterodimer formed between subunits of the Chinese hamster and the murine B form of GPI. AB designates the murine heterodimer. O is the origin. P19A2D signifies differentiated cells derived from P19A2. Lane a, P19A2; b, FdU8B; c, 3T3; d, P19A2D/FdU8B C1; e, P19A2D/FdU8B C4; f, P19A2/3T3 C2; g, P19A2D/3T3 C1


−
+
BB
AB
AA
CB
O
CC
a
b
c
d
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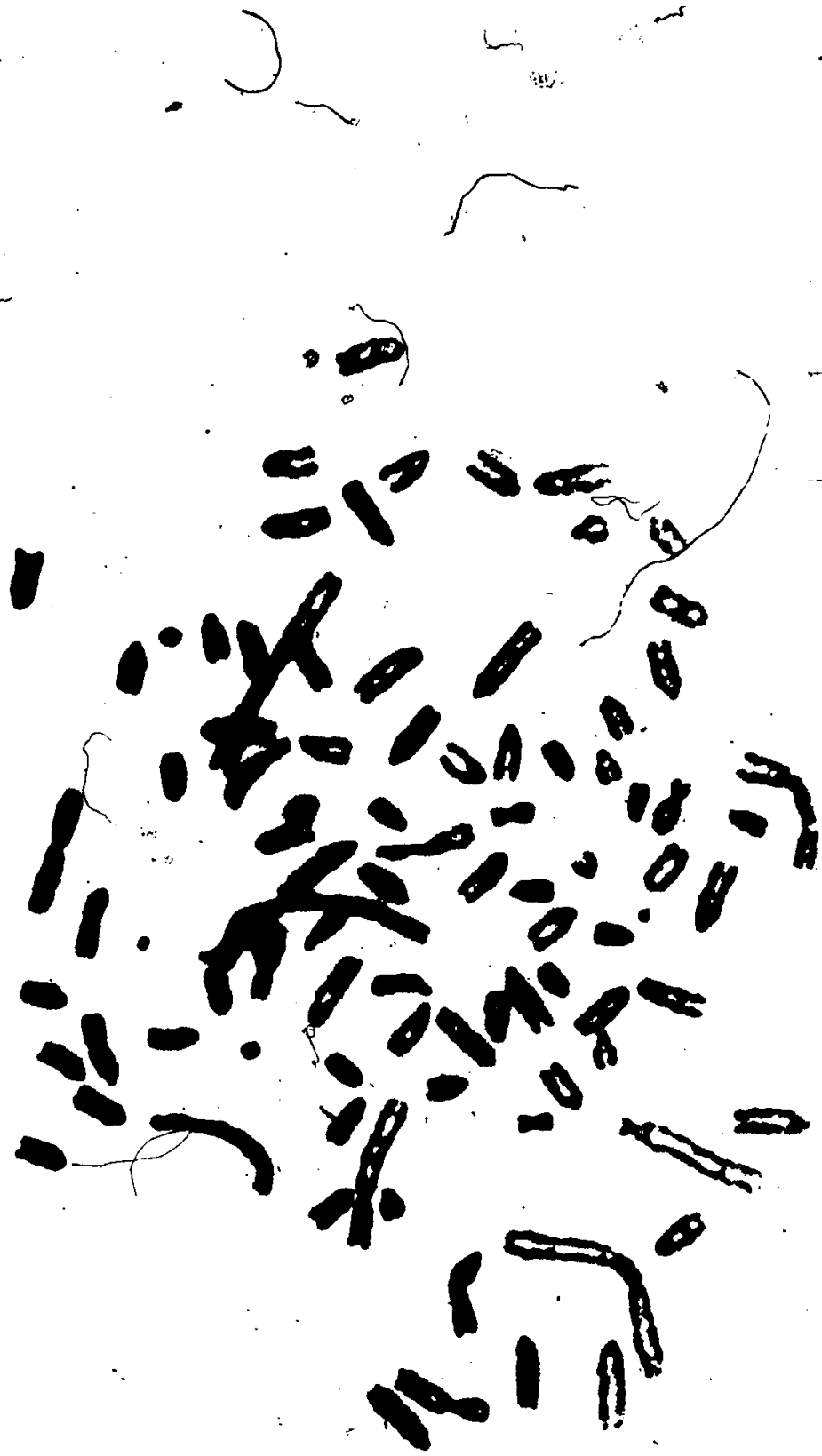
Figure 8: Chromosomes of a hybrid cell derived from a fusion mixture consisting of CHO cells and the differentiated progeny of EC cells. Telocentric chromosomes are of murine origin and all others are of Chinese hamster origin.



The recovery of viable hybrids between CHO cells and the differentiated progeny of EC cells suggests that our difficulty in recovering EC/non-EC hybrids might be due to differences between the pluripotent and differentiated states.

DISCUSSION

We have found that EC cells will readily form heterokaryons with 3T3 fibroblasts, IMR90 fibroblasts, and HeLa cells. Although EC/3T3 hybrids have been produced at a high frequency, we have been unable to recover EC/HeLa or EC/IMR90 hybrids. These results suggest that there is an intracellular incompatibility between EC cells and certain cell types. This incompatibility is reflected in an inability of the heterokaryons to proliferate.

There are precedents for the incompatability of unlike genomes within the same cytoplasm. Johnson and Harris (30) noted that in HeLa/Ehrlich heterokaryons DNA synthesis in HeLa nuclei was suppressed while Ehrlich nuclei were in S phase. Others have shown that EC cells are refractory to infection by the papovaviruses and ecotropic murine C-type viruses (31-34). Segal et al (35) and Segal and Khoury (36) have suggested that the processing of viral pre-mRNA may be the site at which viral infection of EC cells is blocked. We do not know whether the incompatibility between EC cells and certain cell types is analogous to either of the phenomena described above.

We have examined some features of EC/IMR90 heterokaryons. These observations may provide clues as to the nature of the heterokaryon incompatibility. Firstly, EC/IMR90 heterokaryons persist for several days in culture in the presence of HAT and ouabain. Thus, neither a rapid self-destruction

of heterokaryons (such as would be brought about by hydrolytic attack upon either genome), nor a block in transcription, is responsible for our lack of hybrid recovery.

Secondly, the detection of sectored nuclei in cells from EC/IMR90 fusion mixtures suggests that some EC/IMR90 heterokaryons can undergo one cell division. One possible explanation for this is that heterokaryon proliferation is blocked at a specific stage of the cell cycle, for example, S phase. If this were so, heterokaryons formed by the fusion of cells in G_2 would be capable of undergoing mitosis; each daughter cell so formed would then arrest at S phase.

We felt that the inability of some heterokaryons to proliferate might be mediated by a small number of non-EC genes. We attempted to test this possibility by selecting for hybrids between EC cells and subgenomic fragments of non-EC cells. No hybrids were recovered from these experiments.

Although EC/HeLa heterokaryons do not give rise to viable hybrids, we were able to produce proliferating cells which carried both EC- and HeLa-derived chromosomes by prior fusion of either cell type to 3T3 fibroblasts. Thus, the apparent incompatability between EC and HeLa cells is not something which is inherent to the chromosomes themselves.

We have found that the differentiated progeny of EC cells are able to form viable hybrids with CHO cells while pluripotent EC cells are unable to do so. This observation suggests that at least part of the incompatability which

prevents hybrid formation between EC cells and certain non-EC cell types is a reflection of some characteristic of the undifferentiated state.

Pluripotent EC cells are able to form hybrids with 3T3 fibroblasts, but not with CHO or HeLa cells. The differentiated derivatives of EC cells form hybrids with CHO cells in addition to 3T3, but not with HeLa. This finding suggests that within the spectrum of tissue types between pluripotent and specialized cells there may be "stages" of incompatibility. We also note that, with one exception, all of the cells which have been reported to give rise to hybrids with EC cells are derived from rodents. It may be that both evolutionary and developmental factors combine to block hybrid formation between EC cells and other cell types.

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CHAPTER 3

X-CODED ENZYME LEVELS IN MALE AND FEMALE EMBRYONAL CARCINOMA CELLS

CONTENTS:

INTRODUCTION

A female embryonal carcinoma cell line, P10, has recently been isolated in our laboratory (1). I have attempted to determine whether these cells have one or two active X-chromosomes before and after differentiation. This was done by comparing the specific activities of enzymes coded by X-linked and autosomally linked genes in P10 and in a variety of EC cell lines carrying a single functional X-chromosome. Independent tests of X-chromosome activity in P10 were concurrently performed by Dr. Michael W. McBurney and Miss Brenda J. Strutt. These latter tests involved the use of electrophoretic variants of the products of the X-linked gene pgk-1 and an analysis of the DNA replication pattern of the X-chromosomes of P10. Their findings provide strong evidence that P10 contains two active X-chromosomes and that X-inactivation occurs upon differentiation. In view of these facts my results indicate that enzyme specific activities are not reliable indicators of X-chromosome activity in EC cells.

RESULTS

Specific activities of enzymes coded by X-linked and autosomal genes in embryonal carcinoma cells

There is good evidence to suggest that the specific activities of X-coded enzymes are proportional to the number of active X-chromosomes (2,3,4,5,6,7). To determine whether both X-chromosomes in P10 cells are active the specific activities of X-coded and autosomally coded enzymes were measured in P10 and a number of EC cell lines which have a single functional X-chromosome. A description of the cell lines used in this investigation is presented in table 1.

Three enzymes coded by genes linked to the X-chromosome (9) have been measured. These are glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49), phosphoglycerate kinase (PGK, EC 2.7.2.3) and hypoxanthine-guanine phosphoribosyl transferase (HGPRT, EC 2.4.2.8). Two autosomally coded enzymes functionally related to G6PD and HGPRT were also measured. These are 6-phosphogluconate dehydrogenase (6PGD, EC 1.1.1.44) and adenine phosphoribosyl transferase (APRT, EC 2.4.2.7) respectively.

P10 has been shown to carry two active X-chromosomes by independent criteria (1). One might expect the specific activities of G6PD, PGK and HGPRT in P10 to be twice those found in the cell lines which carry a single functional X-chromosome. The specific activities of these enzymes in

TABLE 1

Some Characteristics of the Embryonal Carcinoma Cell Lines Used

	Cell line	Derivation	Chromosome number	Sex chromosomes	Number of active X-chromosomes	Reference
1.	P10	teratocarcinoma	40	XX	two	1
2.	P10S1	clone of P10	40	XX	two	1
3.	P10S1S10	clone of P10S1	40	XX	two	1
4.	P10S1C6	clone of P10S1	40	XX	two	1
5.	P10S1C4	clone of P10S1	39	XO	one	1
6.	P10S1C8	clone of P10S1	40	XO	one	1
7.	OC15S1	clone of OC15	40	XO	one	8
8.	C145f	teratocarcinoma	40	XX	one	1
9.	C86S1	clone of C86	41	XX	one	8
10.	P19	teratocarcinoma.	40	XY	one	1
11.	5'	teratocarcinoma	41	XY	one	1

P10 and other EC cell lines are presented in Table 2. An examination of the data reveals that there was a great deal of variability in enzyme activities between cell lines and between independent extracts of the same cell line. The variability may be a consequence of minor variations in culture conditions although every attempt was made to prepare extracts from cells growing under identical conditions. Despite this variability it can readily be seen that the specific activities of X-coded enzymes in P10 and its karyotypically XX subclones are not twice those of cell lines with a single functional X-chromosome. Particular attention should be given to the values for PGK. It has been shown that both the paternally and maternally inherited pgk-1 alleles are expressed in P10, P10S1, P10S1S10, and P10S1C6 (1). This fact is not reflected in their specific activities. In fact, the highest values are obtained with the male line P19. Only P10S1C6 has a PGK activity higher than any of the controls.

Some of the variability apparent in G6PD and HGPRT specific activities can be compensated by expressing the data as the ratio of the specific activity of X-coded enzyme to that of functionally related autosomally coded enzyme. We might expect that the specific activities of functionally related enzymes would fluctuate to the same extent when changes in metabolic activity take place. Enzyme ratios would thus be more uniform from extract to extract of the same cell line than actual specific activities. Ratios should

TABLE 2

Specific Activities[1] of Five Enzymes in Undifferentiated Embryonal Carcinoma Cells

	Cell line	Sex chromosomes	X-linked enzymes G6PD	PGK	HGPRT	Autosomal enzymes 6PGD	APRT
1.	P10	XX	23±5[2]	410±140	0.95±0.20	24±2	1.00±0.20
2.	P10S1	XX	44±10	500±50	0.91±0.20	47±10	0.98±0.10
3.	P10S1S10	XX	21	300	0.72	30	1.01
4.	P10S1C6	XX	33	660	0.89	45	1.18
5.	P10S1C4	XO	29	560	1.00	29	0.90
6.	P10S1C8	XO	27	600	0.96	29	1.14
7.	OC15S1	XO	35	700	n.d.[3]	38	n.d.
8.	C145f	XX	26±10	560±180	1.29±0.60	15±7	0.82±0.20
9.	C86S1	XX	44	610	n.d.	32	n.d.
10.	P19	XY	73±10	1130±200	1.37±0.30	38±9	0.94±0.20
11.	5'	XY	32±10	650±140	1.14±0.20	19±6	0.72±0.10

1 Specific activities are in nmoles/min/mg protein. Enzyme determinations were performed according to McBurney and Adamson (1976) (8) with the modification that G6PD activity was not corrected for contributing 6PGD activity which was deemed negligable. Protein determinations were performed according to the modified Lowrey method of Hartree (10).

2 Specific activities given with standard deviation are the averages of five to thirteen separate determinations. Values given without standard deviation are the averages of at least two independent assays.

3 not determined

also prove more amenable to comparisons between cell lines. We would expect that cells carrying two active X-chromosomes should have enzyme ratios twice those of cells carrying a single active X.

Histograms of G6PD to 6PGD and HGPRT to APRT ratios for the various cell lines are presented in figure 1. It can be seen that P10 and its XX subclones have ratios which are not twice those of lines with a single functional X-chromosome; in fact, they tend to be lower.

An internal control for these data is available. The aprt gene is linked to chromosome 8. P10S1C8 is trisomic for this chromosome (1). One would predict the HGPRT to APRT ratio of P10S1C8 to be 67% that of its sister clone P10S1C4 which has two chromosomes 8. The value is 77%.

It should be noted that a greater degree of consistency was observed within experiments than between them. Table 3 gives the specific activities and ratios for a number of lines obtained in a single experiment. It can be seen that the range of specific activities is much narrower than in table 2 which compiles the data from a number of experiments done over a long period of time. Although every attempt was made to use fresh reagents, it is possible that not all the components of the reaction mixtures remained perfectly stable over the course of these experiments. This may have given rise to variability in data over the long term.

There is no marked increase in the X-coded enzyme activities of P19, 5' and C145f over those of P10, P10S1 and

Figure 1: Ratios of X-coded to autosomally coded enzyme specific activities in embryonal carcinoma cells. The data are presented as the ratios of the specific activities of X-coded enzymes over those of functionally related autosomally coded enzymes. Where the standard deviation is indicated bar height represents the average of five to thirteen independent determinations. Otherwise bar height represents the average of at least two independent determinations. Bars for lines which carry two active X-chromosomes are grouped at the beginning of each histogram and are stipled. Bar 1, P10; 2, P10S1; 3, P10S1S10; 4, P10S1C6; 5, P10S1C4; 6, P10S1C8; 7, OC15S1; 8, C145f; 9, C86S1; 10, P19; 11, 5'.

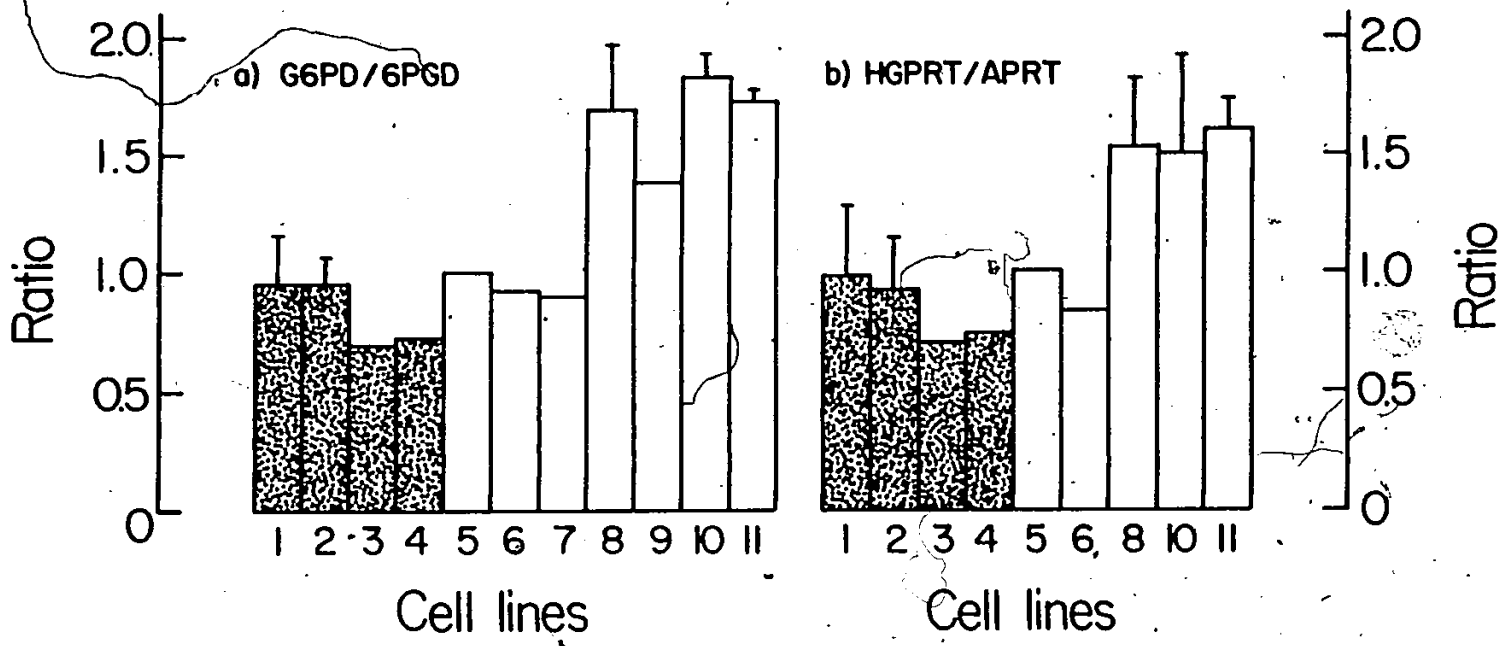

a) G6PD/6PGD
b) HGPRT/APRT
Ratio
2.0
1.5
1.0
0.5
0
1 2 3 4 5 6 7 8 9 10 11
1 2 3 4 5 6, 8 10 11
Cell lines
Cell lines

TABLE 3

Enzyme Levels Determined in a Single Experiment

Cell Line	X-linked enzymes HGPRT	G6PD	PGK	Autosomally linked enzymes APRT	6PGD	HGPRT APRT	G6PD 6PGD
P10	1.36	29	640	1.15	31	1.18	0.93
P10S1	1.22	28	560	0.90	28	1.35	1.00
C145f	0.86	20	710	0.97	21	0.88	0.95
P19	1.37	59	690	1.03	35	1.33	1.68
5'	1.19	26	640	1.23	25	0.96	1.04
OC15S1	0.99	26	840	1.18	33	0.83	0.78

OC15S1 in Table 3, except the G6PD specific activity and G6PD to 6PGD ratio of P19. These results are in contrast with those reported in table 2. Nevertheless, what remains clear and consistent with all other experiments is that P10 and P10S1 have neither specific activities nor enzyme ratios twice those of lines carrying a single functional X-chromosome.

Enzyme levels in differentiated cultures derived from embryonal carcinoma cells

Decreases in X-coded enzyme specific activities of approximately 50% have been reported in developing mouse embryos at a time when X-inactivation is thought to occur (6,7). The specific activities of X-coded and autosomally coded enzymes were measured in differentiated clonal cultures of P10 as a test for the occurrence of X-inactivation. Strong independent evidence that X-inactivation does take place in these lines has been obtained (M. W. M. and B. J. S., personal communication).

Enzyme specific activities in a number of EC cell lines before and after differentiation is presented in table 4. There is clearly no trend towards the halving of X-coded enzyme specific activities in P10 or its subclones upon differentiation. Only with the HGPRT values of P10S1 does this seem to have occurred. The PGK values of P10S1 show a significant increase at two and three weeks after initiation of the differentiation process. Differentiated cultures of

TABLE 4

Specific Activities[1] of Five Enzymes Before And After Differentiation of Embryonal Carcinoma Cells

	Cultures	Sex chromosomes	Weeks[2]	X-linked enzymes G6PD	PGK	HGPRT	Autosomal Enzymes 6PGD	APRT
1.	P10S1[3]	XX	0	18	340	0.93	27	1.03
2.	P10S1[3]	XX	2	22	830	0.68	21	1.19
3.	P10S1	XX	3	16	760	0.43	18	1.19
4.	P10S1C6[3]	XX	0	20	970	0.57	21	1.26
5.	P10S1C6[3]	XX	2	19	930	0.44	20	0.93
6.	P10S1C4[3]	XO	0	19	870	0.63	27	1.38
7.	P10S1C4	XO	2	18	950	0.78	18	1.04
8.	OC15S1	XO	0	37	580	n.d.[4]	38	n.d.
9.	OC15S1	XO	1	19	360	n.d.	24	n.d.
10.	C86S1	XX	0	38	520	n.d.	25	n.d.
11.	C86S1	XX	1	30	470	n.d.	16	n.d.
12.	P19	XY	0	49	860	n.d.	31	n.d.
13.	P19	XY	1	28	680	n.d.	16	n.d.

1 Specific activities are given as nmoles/min/mg protein.

2 This column indicates the number of weeks after initiation of the differentiation process at which enzyme assays were performed. Differentiation in lines numbered 1 to 7 was initiated by culture in suspension. Differentiation in the remaining lines was induced by culturing in 10^{-7} M retinoic acid.

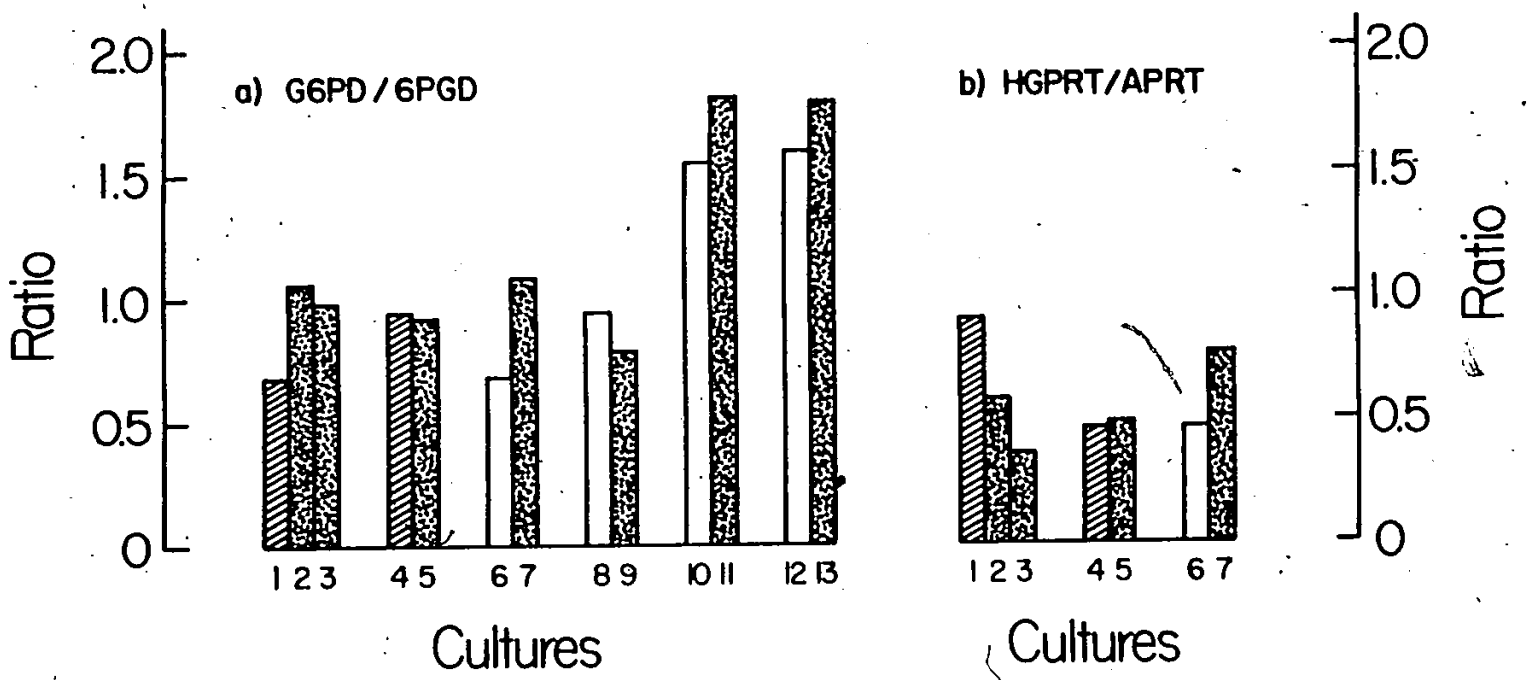
3 These cultures were independently assayed twice. All other cultures were assayed once.

4 not determined

P10S1C6 maintain PGK levels comparable to the undifferentiated state. These results are not consistent with the observation that differentiated cultures of P10S1 and P10S1C6 display a single electrophoretic form of PGK.

G6PD to 6PGD and HGPRT to APRT ratios are presented in figure 2. Again, the only instance in which these values seem to drop by half as differentiation proceeds is for the HGPRT to APRT ratios of P10S1. Concurrent with this decrease the G6PD to 6PGD values rose in P10S1. Both ratios remained stable in differentiating P10S1C6. Moderate fluctuations are observable in most of the control lines. P10S1C4 may display significant increases in both ratios, however the reproducibility of this event has not been tested yet. It is clear from these results that X-coded enzyme levels do not reflect the occurrance of X-inactivation in the female EC cell lines studied.

Figure 2: Enzyme ratios before and after differentiation of embryonal-carcinoma cells. The cell lines and the stages of differentiation at which they were assayed are as given in table 3. Undifferentiated cell lines which are believed to undergo X-inactivation upon differentiation are indicated by diagonal markings. Undifferentiated cell lines which carry a single active X-chromosome have open bars. Differentiated cultures have stipled bars. Bars 1, 2 and 3, P10S1; 4 and 5, P10S1C6; 6 and 7, P10S1C4; 8 and 9, OC15S1; 10 and 11, C86S1; 12 and 13, P19.


a) G6PD / 6PGD
b) HGPRT/APRT
Ratio
2.0
1.5
1.0
0.5
0
1 2 3
4 5
6 7
8 9
10 11
12 13
Cultures

DISCUSSION

Studies on early mouse embryos (5) and the germ cells of XX and XO mice (2) have revealed that the specific activities of X-coded enzymes within cells are proportional to the number of active X-chromosomes carried by those cells. I, therefore, undertook to use X-coded enzyme specific activities to assess whether both X-chromosomes were active in a female EC cell line and subclones derived from it. The results of this investigation revealed that these female EC cell lines did not have twice the X-coded enzyme levels of EC cells which had a single active X-chromosome. However, independent tests of X-chromosome activity have provided strong evidence that the female EC cell lines in question carry two active X-chromosomes. In view of this finding, I interpret my results to suggest that dosage compensation in the absence of X-inactivation has taken place in these female cells. This would require the action of regulatory mechanisms of X-linked gene expression not observed before in pluripotent cells.

I have also observed that the levels of X-coded enzymes of EC cells with two active X-chromosomes show no general decrease upon the differentiation of EC cells with two active X-chromosomes. We have obtained independent evidence through cytogenetic analysis and the electrophoretic detection of

pgk-1 allelic expression that X-inactivation does take place upon the differentiation of P10 and its derivatives. It must be concluded that the specific activities of X-coded enzymes and the ratios of X-coded to autosomally coded enzyme activities are not reliable indicators of X-inactivation in the EC cell lines studied.

X-coded enzyme levels fluctuate widely in and between undifferentiated and differentiated cultures regardless of whether they were derived from EC cells having one or two active X-chromosomes. This is in marked contrast to the 50% decrease in X-coded enzyme levels inferred to take place in developing female mouse embryos, due to X-inactivation (6,7). EC cell lines are quite different in the range and extent to which they will form various differentiated cell types. The proportion of cells in differentiated cultures which are mitotically active should also vary depending on cell types formed and cell confluency. The embryo, on the other hand, has a reproducible array of mitotically active tissues at given stages of embryogenesis. The variability of differentiated cultures derived from EC cells may impose a background of enzyme activity fluctuations against which changes due to X-inactivation can not be observed.

The results of this investigation are in contrast with those of Martin et al (11) who found X-coded enzyme levels to be twice as high in karyotypically XX EC cell lines as in XO. These levels dropped by half during the course of

differentiation of the XX line. These EC cells were parthenogenetically derived whereas ours were obtained by embryo transplantation. More karyotypically XX EC lines would have to be examined before a relationship could be drawn between X-coded enzyme levels and the manner in which teratocarcinomas are derived.

CONCLUSIONS

It is clear from this investigation that X-linked gene expression in EC cells is subject to a variety of influences. Enzyme levels are therefore not reliable indicators of the number of active X-linked gene copies per cell in the EC lines studied. Factors which affect X-chromosome expression in EC cells may be resident in the heterogeneity between EC cell lines, the continuous culture of EC cells which carry two active X-chromosomes, a modulating effect of inactive sex-chromosomes, and the spectrum of tissue types produced in differentiating cultures. It should be noted that this investigation is not the first to report anomalous findings on X-linked gene activity (12,13).

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CHAPTER 4

DISCUSSION AND SPECULATION

CONTENTS:

DISCUSSION AND SPECULATION

In this thesis I reported the results of two projects which were designed to assess X-chromosome activity in EC cells. In the main project I attempted, without success, to produce EC cells carrying foreign X-chromosomes by means of cell hybridization techniques. Although 3T3/HeLa hybrids formed tri-hybrids with EC cells, I was unable to produce EC cells carrying the HeLa X-chromosome by fusion to 3T3/HeLa microcells.

Heterokaryon Incompatibility

In the course of these experiments I attempted to determine why it was difficult to recover hybrids with EC cells. The results of this investigation, presented in chapter two, have proven very interesting. The recovery of hybrids between CHO cells and the differentiated progeny of EC cells suggests that heterokaryons formed between EC cells and some non-EC cell types do not proliferate due to some form of incompatability between the pluripotent and differentiated states.

I have not determined the exact nature of this heterokaryon incompatability. Because I have observed sectored nuclei in C145A12/IMR90 fusion mixtures, it is possible that some heterokaryons can undergo at least one cell division. This suggests that heterokaryon/hybrid proliferation may be blocked at a specific stage of the cell

cycle. Thus, if entry into S phase was inhibited in the heterokaryons, those formed by the fusion of cells in G_2 could presumably go through one mitosis.

It is possible to speculate on a number of sources of heterokaryon incompatibility.

1. It may be that the entry into S phase of one or both parental nuclei is inhibited in EC/non-EC heterokaryons. Two ways in which this might arise can be envisaged. (a) There is some evidence to suggest that mammalian cells may have more than one set of initiation factors and initiation sites for DNA replication (1). It may be, for example, that EC and some non-EC cell types employ different initiation factors and initiation sites. Initiation sites which are not used may be masked due to chromatin conformation. If only one of the parental initiation factors are synthesized in the heterokaryons, then DNA of the other parent would not be replicated. This might result in the failure of the heterokaryons to enter mitosis or in abortive mitosis. Some cell types may form viable hybrids with EC cells because they employ the same initiation factors as EC cells, or because, although they employ different initiation factors, both are expressed in the heterokaryon, or because initiation sites recognized by EC initiation factors are not masked.

(b) 2-5A synthetase is an enzyme which is induced in many cell types by treatment with interferon (2). The product of this enzyme, 2-5A (an oligonucleotide with 2' to 5' linkage), is a potent inhibitor of protein synthesis.

Dephosphorylated 2-5A has been shown to inhibit DNA synthesis and cell growth (3). In the absence of interferon treatment various cell types have been shown to differ by a thousand fold in 2-5A synthetase activity (2). It may be that cell types with high base-line levels of 2-5A synthetase are unable to form viable hybrids with EC cells. EC cells are not capable of an interferon response (4) and have high levels of alkaline phosphatase (5). Alkaline phosphatase could act on 2-5A to produce the dephosphorylated derivative. This product could act to block proliferation in heterokaryons formed between EC cells and those non-EC cell types which have high levels of 2-5A synthetase. The 2-5A synthetase activity of the non-EC cells used in my experiments are not known. If, however, this hypothesis is correct, then raising the levels of 2-5A synthetase in 3T3 cells should block the formation of EC/3T3 hybrids.

2. Another mechanism which could form a basis for heterokaryon incompatibility involves a difference in the RNA splicing machinery of pluripotent and differentiated cells. Small nuclear (sn) RNAs are components of ribonucleoprotein particles (RNPs) (6). It has recently been shown that the intron-exon junction sequences of genes from a variety of sources show significant complementarity to a snRNA of mouse cells known as U1 (7). Furthermore, RNPs are found associated with hnRNA in cell extracts (7). On the basis of this evidence it has been suggested that RNPs form the RNA splicing complexes of cells and that the snRNAs serve to

align the splicing enzymes at the intron/exon junctions (6,7).

Differences between EC and non-EC cells in snRNAs available for splicing might account for heterokaryon incompatability. It seems clear that gene expression could be regulated at the level of RNA splicing. The exposure of nuclei to novel snRNAs through cell hybridization could upset this regulation by allowing normally unspliced messages to be processed. The ultimate effect of the gene products of mature transcripts generated in this manner might be the induction of a senescent state. It could be that some cell types share a common array of snRNAs with EC cells, and thus are able to form viable hybrids with them. For this reason, and for their possible role in the regulation of gene expression, it would be of interest to compare the spectrum of snRNAs between EC cells and various differentiated cell types.

All the genes which have been shown to contain sequences complementary to U1 snRNA are either expressed only in differentiated cells, or are derived from the papovaviruses which infect differentiated cells. It may be that U1 snRNA is specific to differentiated cells. An absence of U1 RNA in EC cells would explain why spliced SV40 mRNA can not be detected in SV40-infected EC cells. Such a finding would also suggest that U1 snRNA might have a role in the regulation of differential gene expression.

I have speculated here on three possible mechanisms of heterokaryon incompatability. It would be of interest

to define the nature of this phenomenon exactly. Knowledge of the molecular basis for the intracellular incompatability between pluripotent and differentiated cells might provide clues to the mechanisms by which the pluripotent state is maintained and commitment to a developmental pathway is achieved. In attempting to determine the differences which block viable hybrid formation between EC cells and some non-EC cell types it might be useful to define when in the differentiation process the progeny of EC cells are able to form hybrids with CHO cells. This could be done by carrying out fusion experiments using EC cells which have been differentiating for progressively longer periods of time. One could then attempt to determine whether a specific metabolic change, such as a decrease in alkaline phosphatase activity or the appearance of interferon inducibility, is coincident with the ability to form hybrids with CHO cells. Such a correlation might help to define the nature of the heterokaryon incompatability at the molecular level.

Female EC Cells Carrying Two Active X-chromosomes

The second project presented in this thesis concerned the quantitation of the specific activities of X-coded enzymes in male and female EC cell lines. This investigation revealed that EC cells with two active X-chromosomes (8) did not have twice the specific activities of X-coded enzymes as EC cells with a single active X-chromosome. Although EC

cells with two active X-chromosomes were shown to undergo X-inactivation upon differentiation (M. W. McBurney and B. J. Strutt, personal communication), X-coded enzyme levels in these differentiated cells did not drop by half accordingly.

These results were surprising because other workers have shown that X-coded enzyme specific activities are proportional to the number of active X-chromosomes in mouse oocytes (9,10), early mouse embryos (10-14), and EC cells (15). Clearly, the female EC cells examined here have undergone dosage compensation in the absence of X-inactivation. This suggests that EC cells are able to employ mechanisms other than X-inactivation for the regulation of expression of X-linked genes. If so, it is reasonable to expect that these mechanisms are of functional significance to the mouse. Presumably X-linked gene expression is controlled in various cell and tissue types in response to the metabolic demands of those cells (16). Such a metabolic regulation of X-linked gene expression may be operative in the female EC cells of this investigation. Under this form of regulation, X-coded enzyme specific activities would not be expected to drop by half upon differentiation. Rather, any changes in enzyme levels would be a response to the changed metabolic requirements of the differentiated cells. The seemingly random fluctuations in X-coded enzyme levels observed in the differentiating cultures of EC cells may reflect this situation.

Female EC cells of parthenogenetic derivation have

been found to have specific activities of X-coded enzymes twice those of karyotypically XO EC cells (15). The female EC cells of this investigation were derived by embryo transplantation (8). More female EC cells of embryonic and parthenogenetic origin should be examined in order to determine whether the mode of teratocarcinoma production is correlated with the level of X-linked gene expression in female EC cells. If so, it may suggest that there are physiological differences in the cells of origin of teratocarcinomas produced in one or the other manner.

It is not clear whether embryo-derived teratocarcinomas originate from embryonic ectoderm or from germ cells like the parthenogenetically derived tumours (5). The fact that the female EC cells of this investigation have two active X-chromosomes suggests that they were derived from germ cells. This is because, although both primordial germ cells and cells of the embryonic ectoderm inactivate an X before the time of embryo transplantation, early germ cells reactivate this X prior to the onset of meiosis (16). This is reflected in a two-fold increase in the specific activities of X-linked enzymes in the oocytes of XX mice over those of XO mice (16).

It has not, however, been shown that a two-fold increase in X-linked gene expression is concurrent with reactivation of the inactive X-chromosome. Thus, it may be that X-linked gene expression is metabolically regulated in germ cells both before and for some time period after X-

chromosome reactivation. Under metabolic regulation X-coded enzyme levels would be expected to remain the same before and after reactivation of the X, until such time as an alternate regulatory mechanism imposed a two-fold increase in X-linked gene expression. EC cells derived from germ cells prior to this two-fold increase (but after X-chromosome reactivation) might escape developmental cues signaling heightened X-linked gene expression and continue to be metabolically regulated. These EC cells would not have X-coded enzyme levels twice those of EC cells having a single X-chromosome. On the other hand, parthenogenetically produced female EC cells are derived from germ cells which have already undergone a two-fold increase in X-linked gene expression. Therefore, parthenogenetically derived female EC cells with two active X-chromosomes would be expected to have twice the specific activities of X-coded enzymes as karyotypically XO EC cells. This hypothesis would reconcile my results (chapter three) with those of Martin et al. (15) under a common scheme.

One prediction of the hypothesis is that it should not be possible to parthenogenetically produce female EC cell lines which have two active X-chromosomes and X-coded enzyme levels comparable to those of XO EC cells. Furthermore, it suggests that germ cells are competent to form teratocarcinomas over a range of developmental stages demarcated by the activity of the X-chromosome. It also suggests that a two-fold increase in X-linked gene expression in XX cells

over XO cells is something which is dependent not only on the active state of both X-chromosomes, but also on regulatory mechanisms peculiar to cells of a pluripotent nature, that is, germ cells, early embryonic cells, cells of the embryonic ectoderm, and EC cells.

Despite peculiarities in X-linked gene expression, it is clear that EC cells with two active X-chromosomes can be obtained from embryo-derived teratocarcinomas (8). Furthermore, these cells will undergo X-inactivation upon differentiation (M. W. McBurney and B. J. Strutt, personal communication). Progress towards an understanding of the phenomenon of X-inactivation has been slow to this point, largely due to limitations imposed by the mammalian embryo. The acquisition of female EC cells which will undergo X-inactivation upon differentiation should remove these restrictions. It should now be possible to make inroads towards defining the molecular mechanisms by which X-inactivation takes place, and establishing a chronology for X-inactivation in the differentiation process.

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