Properties of Teratocarcinoma Somatic Cell Hybrids

Richard A. Miller
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PROPERTIES OF TERATOCARCINOMA SOMATIC CELL HYBRIDS

A Dissertation
Presented to the Faculty of the Graduate School
of
Yale University
in Candidacy for the Degree of
Doctor of Philosophy

by
Richard A. Miller
December, 1976
ABSTRACT

PROPERTIES OF TERATOCARCINOMA SOMATIC CELL HYBRIDS

Richard A. Miller

Yale University 1977

This dissertation describes the production and characterization of two series of somatic cell hybrids. PCT hybrids result from the fusion of mouse thymocytes with PCC4azal embryonal carcinoma cells (pluripotent cells derived from a mouse teratocarcinoma). PCF hybrids arise from the fusion of PCC4azal with FBU, a line of Friend erythroleukemia cells.

These hybrid cells resemble their embryonal carcinoma parents in many ways. They look like PCC4azal by phase contrast and electron microscopy. Like embryonal carcinoma cells, they have high levels of alkaline phosphatase. They are resistant to infection by a parvovirus, MVM, which can infect many mouse cell lines, but not embryonal carcinoma cells. The hybrids also produce tumors which contain a variety of cell types, including derivatives of all three of the classical germ layers. The most highly-differentiated tumors produce mouse α-fetoprotein.

Traits characteristic of the non-pluripotent parental cell line, such as Thy-1 antigen in thymocytes, and acetylcholinesterase in Friend cells, are not expressed in the hybrid cells. PCC4azal and FBU also differ in their pattern of lactate dehydrogenase isozymes, and the PCF cells resemble PCC4azal in this respect, too. PCF populations, unlike those of FBU, do not contain hemoglobinated cells.

At least some of the cells in PCT populations do, however, exhibit H-2
antigens, which are expressed only at very low levels, if at all, in the
PCC4azal parental cells.

These experiments show that although embryonal carcinoma cells can,
under appropriate developmental conditions, give rise to thymocytes and
erthroblasts, the genome of the pluripotent cell does not respond to
the signals that these two differentiated cell types use to maintain
their stable epigenetic state. On the contrary, pluripotent cells may
contain a molecular mechanism able to "reset" the genetic apparatus of a
differentiated cell.
To Bernie and Selma
ACKNOWLEDGEMENTS

I have had a lot of help from a lot of people while working on this thesis, and Frank Ruddle is surely first among them. I am grateful for his manufacturing an environment in which research can thrive, for pointing me in the right direction within that environment, for letting me wander down a few back alleys but also for pointing out the dead-end signs; in short, for supplying the strategic and tactical guidance that the naive enthusiast so badly needs. I owe him a great deal.

Many "teratomologists" have given me generously of their time, their expert knowledge, and their thrill-of-the-chase. Roy Stevens, especially: having invented teratomas, Roy now takes an amazing amount of time from his own busy research schedule to coach us newcomers. To Mike Sherman, friend and collaborator, and to Karen Artzt, immunology coach extra-ordinaire, I also owe large debts. Thierry Boon, Francois Jacob, Robert Jacoby, Hedwig Jakob, Barbara Knowles, Arnie Levine, Nancy Ruddle, Seung-il Shin, and Mette Strand were all of help in ways ranging from gifts of key cell lines to (Nancy's) lessons in how to tell the thymus from the salivary glands.

A number of others actually did some of the work. Elizabeth Nichols carried out the starch gel analyses. Pat Yao made the electron micrographs. Dave Holbrook, Bill Sacco, and Andy Skolnick prepared many of the final photographic prints, and Jim Brosius did the original drawings. Linda Dohrman and Rachel Briggs prepared histological sections from the fixed tumors. Mae Reger typed faster and more accurately than I could think.

Drs. Edward Adelberg, Xandra Breakefield, Bob Cone, Jerry Eisenstadt, Joseph Gall, Tom Gelehrter, Doug Kankel, and Dave Ward have each, at one
time or another, supplied cogent advice as members of a constantly evolving thesis committee. Carolyn Slayman, my infant department's DGS, has played Vergil to my Dante in this hot place.

Since I first joined the Ruddle lab, it has been host to 83 (!) other fellow travelers, approximately all of whom have been sources of many boons, from feeding the cells to buying the pizza to rectifying the seminar. I hope the other 76 of you will forgive me if I limit myself, in print, to five special cases: Vinnie Salerno, Burt Dorman, Hans-Peter Bernhard, Raju Kucherlapati, and Susan Elsevier.
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A Note on Terminology

I wish to state here how I intend to use certain terms often employed by other writers in diverse ways.

Teratoma, teratocarcinoma, embryonal carcinoma, embryoma: I intend to use these terms in the ways suggested by Damjanov and Solter (1974), and by Stevens and Pierce (1975). Teratocarcinomas are transplantable tumors which contain cells of many different tissue types. Some of the cells in teratocarcinomas are embryonal carcinoma cells, which are thought to render the tumor transplantable, and are thought to be the cell type from which all the other types are derived. The word "teratoma" is commonly used in two senses: first, to denote a teratocarcinoma-like tumor which is not, however, transplantable (because it contains no embryonal carcinoma cells); and second, as a lumping term which includes both the transplantable and non-transplantable varieties. I shall use the word in both senses, and use the phrase "benign teratoma" when clarity requires it. Embryomas are growths which, like teratomas, contain a variety of cell types, but do not have the potential to grow continuously and never have contained embryonal carcinoma cells. Embryomas can be produced experimentally by the ectopic implantation of mouse embryos after the developmental stage at which transplantable teratocarcinomas can last be produced.

Embryo versus conceptus: strictly speaking, the conceptus includes both the embryo proper and the extra-embryonic membranes. I shall, however, use the word "embryo" as synonymous with "conceptus," and the phrase "embryo proper" when distinction from extra-embryonic membranes is needed.

Malignant, transplantable, transformed, tumorigenic: to call tumors transplantable, and cells tumorigenic, gives no one any trouble, since the
terms are operational. I shall avoid the term "transformed" unless it is
clear what is being transformed by what. It is, for example, by no means
clear that embryonal carcinoma cells, although tumorigenic, have undergone
any sort of transformation. Some people use "malignant" to mean "meta-

crasiasizing," and others as a synonym for "transplantable;" I shall avoid
its use.

Pluripotent, determined, differentiated: the confusion these terms
evoke reflects, in part, our ignorance of the decision-making process within
the embryo. I will restrict the use of "pluripotent" to those cells which can
give rise to several kinds of fundamentally different cells. Friend cells are
not, in this sense, pluripotent, even though they can give rise to normoblast-
like cells different in many ways from ordinary Friend erythroblastoid cells.
I consider a cell "determined" or "committed" when it has entered into that
ill-defined state from which it can, under ill-defined "normal" conditions,
produce only the limited variety of cell types towards which it has just become
determined. We know, since leukocyte albumin genes can be activated under some
conditions (e.g. Darlington et al., 1974), that abnormal conditions can undo
the effects of determination. I will not fall for the etymological cavil
that says "all cells are differentiated" just because they all look different.
A differentiated cell, as I shall use the term, is one that has attained a
state in which it remains stable within limits defined by the cell cycle (if
still dividing) and modulating influences (e.g. hormones). A stem cell, all
of whose progeny are either stem cells or (e.g.) epidermal cells, is determined
but not yet differentiated. In the past, many somatic cell geneticists have
called some particular cell line (usually fibroblastoid) "undifferentiated"
because these cells were unlike the differentiated cell line with which they
were being compared or fused. I do not use the term in this way; when one
fuses a fibroblast with an embryonal carcinoma cell, it makes little sense to
call the former cell undifferentiated.
LIST OF ABBREVIATIONS

AChE: acetylcholinesterase  
APase: alkaline phosphatase  
C': complement  
DMSO: dimethyl sulfoxide  
DVME: Dulbecco-Vogt modified Eagle's medium  
DVME-FC: DVME with 10% fetal calf serum  
DVME-HAT: DVME with HAT additives  
DVME-o: DVME without any additives (except antibiotics)  
ECC: embryonal carcinoma cell  
EDTA: ethylene diamine tetra-acetate  
FBU: a line of BrdU-resistant Friend erythroleukemic cells  
FCS: fetal calf serum  
GPI: glucose phosphate isomerase  
HAT: a mixture of hypo-anthine, aminopterin and thymidine; concentrations are given in the text  
IDH: isocitrate dehydrogenase  
LDH: lactate dehydrogenase  
MOI: multiplicity of infection  
MPC: mesectodermal precursor cell  
MVM: minute virus of mice  
PCC4azal: an azaguanine-resistant embryonal carcinoma cell line  
PCF: a line of PCC4azal x FBU hybrids  
PCT: a line of PCC4azal x thymocyte hybrids  
PFU: plaque-forming units  
PCC: primordial germ cells  
PGM: phosphoglucomutase
"I'm worried about Paul," Martha said.

"Hasn't he done this kind of thing before?" Helen essayed a reassuring laugh. "I'd gathered this was more or less normal with him and Nicole."

"Yes, but...it was different when she was with him. She never let him go on like this. It's been over two weeks! I don't know what to do--he won't let me help him."

Gordon, who was doing sit-ups, grunted for attention. "Probably the best thing that could have happened," he interjected. "I mean, getting involved with his work. Martha ought to know that."

7:00 a.m., October 24. Tape recording (Paul Holliston):

"Administering 500 cc of Methotrexate...a risk, because of its high addictive quality. If Methotrexate doesn't arrest the rapid growth, I have no choice but to transfer the subject to the University Research Clinic. I can no longer cope..."

--From Embryo, by Louis Charbonneau. Based on the screenplay by Anita Doohan and Jack W. Thomas.
PART 1A: Somatic cell hybrids as tools with which to study patterns of gene expression

The central theme of this dissertation is that there are rules which specify the patterns of gene expression which a cell can adopt and which govern the changes from one pattern to another, and that one can obtain insights into these rules by characterizing somatic cell hybrids whose parents differ epigenetically.

This theme is by no means new. Indeed, the earliest experiments Harris performed after he and Watkins (Harris and Watkins, 1965) introduced Sendai-virus-mediated cell fusion as a means of promoting somatic cell hybrid formation were based on such expectations. In these studies (reviewed in Harris, 1970), Harris and his collaborators showed that when heterokaryons were formed between two cell types, of which only one made RNA (or both RNA and DNA), nucleic acid synthesis in the nucleus of the less active parent was induced to match the pattern of the other parent. Since these reports, a large literature has accumulated based on the characterization of hybrids and heterokaryons whose parents differed epigenetically. I intend to draw upon this literature only to illustrate the principles discussed below; more comprehensive reviews are available (Davis and Adelberg, 1973; Davidson, 1974).

The work of Davidson and his collaborators on melanoma x fibroblast hybrids not only presented the first detailed study of
"intertypic" hybrid cells (i.e., hybrids whose parents were of different epigenetic types), but also suggested two generalizations which later work has, for the most part, supported. An early report (Davidson et al., 1966) showed that Syrian hamster melanoma x mouse L cell fibroblast hybrids were unpigmented; later work (Davidson, 1972) demonstrated that hybrids formed between the same fibroblast line and a melanoma line related to the first one but containing twice the chromosome number were often pigmented.

One generalization suggested by these findings is that L cell traits predominate in hybrids formed by fusing L cells with other kinds of cells. Many intertypic fusions have involved L cells or other aneuploid fibroblastoid lines with long histories of growth in vitro. During the course of such a long and sometimes quixotic selection for cells which grew well under artificial conditions (see Ham (1974) for a resumé of the trials of the L cell line), inadvertant selection may also have occurred for cells unusually able to dominate intertypic fusions with parents less well adapted to culture. In hybrids between these aneuploid fibroblasts and other cell types, extinction of the tissue-specific phenotypes of the nonfibroblast parent often takes place. A selection of such reports is shown in Table 1-1. This table also shows, however, that while the "rule" of fibroblast extinction is consistent with the results of many cell fusion experiments, much of the data is equally consistent with the hypothesis that relative chromosome input ("gene dosage") determines hybrid properties. A "pure" test of this "fibroblast extinction" rule would require that one fuse a given nonfibroblast line with a number of other kinds of cells, including aneuploid fibroblasts, while
TABLE 1-1
REPRESENTATIVE FIBROBLAST HYBRIDS

<table>
<thead>
<tr>
<th>Other Cell</th>
<th>Property</th>
<th>Chromosomes</th>
<th></th>
<th></th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Fibro.</td>
<td>Other</td>
<td>Results</td>
<td></td>
</tr>
<tr>
<td>Melanoma</td>
<td>melanin</td>
<td>52</td>
<td>51</td>
<td>extinction</td>
<td>Davidson et al., 1966</td>
</tr>
<tr>
<td></td>
<td></td>
<td>52</td>
<td>91</td>
<td>retention</td>
<td>Davidson 1972</td>
</tr>
<tr>
<td>Glial</td>
<td>S-100 protein</td>
<td>53, 75</td>
<td>42</td>
<td>extinction</td>
<td>Benda and Davidson, 1971</td>
</tr>
<tr>
<td></td>
<td></td>
<td>53</td>
<td>2x 42</td>
<td>*extinction</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GPDH (baseline)</td>
<td>53</td>
<td>42</td>
<td>extinction</td>
<td>Davidson and Benda, 1970</td>
</tr>
<tr>
<td></td>
<td></td>
<td>53</td>
<td>2x 42</td>
<td>retention</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GPDH (induced)</td>
<td>53</td>
<td>42</td>
<td>extinction</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>53</td>
<td>2x 42</td>
<td>*extinction</td>
<td></td>
</tr>
<tr>
<td>Pituitary</td>
<td>Growth hormone</td>
<td>2x 50</td>
<td>73</td>
<td>extinction</td>
<td>Sonnenschein et al., 1971</td>
</tr>
<tr>
<td>Renal</td>
<td>Esterase-2</td>
<td>45</td>
<td>73</td>
<td>*extinction</td>
<td>Klebe et al., 1970</td>
</tr>
<tr>
<td>Neural</td>
<td>various</td>
<td>52</td>
<td>90, 142</td>
<td>retention</td>
<td>Minna et al., 1972</td>
</tr>
<tr>
<td></td>
<td></td>
<td>47</td>
<td>92</td>
<td>(some props.)</td>
<td>McMorris and Ruddle, 1974</td>
</tr>
<tr>
<td>Thymic</td>
<td>Thy-1 antigen</td>
<td>50-55</td>
<td>ca. 40</td>
<td>extinction</td>
<td>see Table 1-2</td>
</tr>
<tr>
<td>Friend</td>
<td>globin or its mRNA</td>
<td>45-70</td>
<td>37-40</td>
<td>extinction</td>
<td>see Table 1-3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22 (a)</td>
<td>37-40</td>
<td>retention</td>
<td></td>
</tr>
</tbody>
</table>

NOTES: * indicates an exception to the "gene dosage hypothesis"
(a) clones which segregate nearly all their human fibroblast chromosomes also make globin mRNA.
Data on liver cells has been omitted; see Darlington and Ruddle (1975) for a review.
The work on Friend and thymic cells has been synopsized; see text for greater detail.
controlling for the effects of gene dosage. No such set of experiments has been reported.

"Pure" tests of the effects of gene dosage on hybrid character have been carried out. In addition to the work of Davidson (1972), and the work of Fougère et al. (1972) on similar melanoma hybrids, the studies of Davidson and Benda (1970) on glycerol-3-phosphate dehydrogenase in glial-fibroblast hybrids support the idea that histotypic functions extinguished in a set of 1:1 hybrids can nonetheless appear in 2:1 nonfibroblast:fibroblast combinations. The data of Peterson and Weiss (1972) on albumin expression in hepatoma hybrids suggest similar conclusions.

Studies of intertypic hybrids have demonstrated four other phenomena, each of which imposes its own constraints on molecular models of gene regulation. The first I want to mention among these is the finding of Peterson and Weiss (1972) and of Darlington et al. (1974) that a gene which is not expressed in a particular parental cell type can be activated when this parent is fused to a cell in which the gene is expressed. In the first of these studies, the albumin gene of a mouse fibroblast was shown to have been "turned on" by fusion with (probably two) rat hepatoma cells. In the other, human albumin production was demonstrated in a mouse hepatoma x human leukocyte hybrid, in which the hepatoma genome was again at the 2S level. These studies do not explain how hepatoma cells maintain their albumin-producer status nor how leukocytes and fibroblast maintain their nonproducer state, but do prove that subversion of the latter mechanism by the former is possible.
A second phenomenon, that of production, by a hybrid, of molecules not produced by either parental cell type, is illustrated by the demonstration (McMorris and Ruddle, 1974) of choline acetyltransferase in a mouse neuroblastoma x human fibroblast hybrid, and by the demonstration of morphine receptors in neuroblastoma x glioma hybrid cells (Klee and Nirenberg, 1974). It is possible in each case that the property in question could have been present in a small proportion of the neuroblastoma population, and that one of these few cells may have been "captured" by hybrid formation; most hybrid clones in the series studied do not show the "new" activity.

The third phenomenon I wish to mention is that of dissociation, in a hybrid, of differentiated properties which occur together in all the cells of one of the parental populations. Minna et al. (1972), for example, have examined a series of neuroblastoma x fibroblast hybrid lines to see which of the several distinct neuroblastoma marker phenotypes were expressed. They found some hybrid clones in which many of the neural properties were present, and others in which only a subset could be demonstrated. Some of the traits (e.g. action potential production) seemed to be present only in hybrids which already possessed others (e.g. acetylcholinesterase). Tests of a larger series of hybrids might have provided these authors with exceptions to their proposed hierarchy of markers. The hierarchy suggested by their results may, however, be relevant to the sequence of transitions among epigenetic states which occurs in developing neural tissues. An analogous experiment by Szpirer and Szpirer (1975) has shown that mouse hepatoma x mouse or rat fibroblast hybrid cells can retain the ability to secrete transferrin and C3 (third component of complement, secretion of which
is characteristic of the hepatoma cells) but lose the capacity to produce albumin and α-fetoprotein. These "dissociation" experiments begin to suggest the extent to which particular differentiated phenotypes are dependent on others with which they are usually associated.

The fourth lesson I wish to abstract from the library of intertypic fusions is that chromosome loss can be accompanied by re-expression of a differentiated phenotype which had been extinguished in the original hybrid cells. The two studies most often quoted in this context are the work of Klebe et al. (1970b) on reappearance of a kidney-specific esterase in segregating mouse renal adenocarcinoma x human fibroblast hybrids, and that of Weiss and Chaplain (1971) on reappearance of tyrosine aminotransferase inducibility in rat hepatoma x rat liver epithelial cell hybrids. A further example is provided by the finding of Kao and Puck (1972) that expression of a group of Chinese hamster esterases was dependent on retention of a single human chromosome in a set of hamster-human hybrids.

What do these studies tell us about control of gene expression in normal, i.e. nonhybrid, cells? Davis and Adelberg (1973) have argued convincingly that one cannot draw clean conclusions about molecular mechanisms from the sorts of results discussed above. Extinction of melanin synthesis in melanoma-fibroblast hybrids, for example, might be brought about by dilution of a gene activator characteristic of melanoma cells, presence of extra repressor in fibroblast cells, or any of numerous other effects, not all of which involve transcriptional control of sets of specific genes.

One can, however, draw a few firm conclusions, provided one is willing to forswear explanation at the molecular level. The gene dosage
results suggest that whatever molecules mediate epigenetic status are not present in very great excess over the concentration required to exert their influence. The experiments which demonstrate activation of the albumin gene in leukocytes and fibroblasts show a) that cells whose ancestors long ago committed themselves away from hepatic differentiation have neither lost nor permanently inactivated their liver-specific genes; b) that hepatoma cells not only possess the means to maintain their own phenotype through mitosis, but also the means to activate liver-specific genes in at least some other cell types; and c) that these signals can, at least in some cases, work across species barriers. The studies of dissociation, in hybrids, of genes ordinarily expressed together provide insights into the dependencies of these genes on one another, and on the hypothetical loci responsible for co-ordination among the markers which we can measure. The work on re-appearance, upon segregation, of extinguished tissue-specific markers establishes both that these markers can be carried for many generations in a latent, alert-but-unexpressed state, and that individual chromosomes can mediate the extinction of genes on other chromosomes, even across species. This last result is particularly intriguing: if re-appearance of, for example (Weiss and Chaplain, 1971), TAT inducibility truly depends on the loss of a particular chromosome (or set of hemologues) from the "extinguisher" parent, this suggests that transfer of "extinguisher" status to the corresponding chromosome in the hepatic cell's genome does not take place. Clarification of this important question will require extension to other sets of hybrids, and, especially, rigorous karyotypic analysis.
One cannot yet predict the phenotype of hybrid cells from a knowledge of their parents, although past experience suggests that such a prediction must take into account gene dosage, and suggests, perhaps, that aneuploid cells long since selected for vigorous growth in culture might exert unusual influence over less well-adapted cells. Cells of a particular epigenetic type (e.g. skin fibroblasts, or erythrocytes, or blastomeres) might well be unusually able to dominate intertypic hybrids, or unusually susceptible to influence; but evidence bearing on this question is so far scant.

PART IB: Hybridization of thymus cells (and related studies with thymic leukemias)

Thymus-specific properties have been studied by somatic cell hybridization in several laboratories, but mouse thymocytes per se were used in only one of the five reports listed in Table 1–2.

Two reports (Hyman and Kelleher, 1975; Liang and Cohen, 1975) have shown that when L cells are fused with mouse lymphoid cell lines which carry the thymus-marker antigen Thy-1, hybrids can be produced in which Thy-1 is no longer detectable. In the study of Liang and Cohen (1975), however, the hybrids were shown to carry another thymic antigen, Tla. The L cell line used by Hyman and Kelleher contained about twice the amount of chromosomal material as did their lymphoma parent; Liang and Cohen did not report any karyotypic data.

Namba and Waksman (1975) have shown that when rat thymocytes are fused with mouse lymphoblast cells, the hybrids can retain two thymic phenotypes: they exhibit a rat thymocyte surface antigen, and produce

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TABLE 1-2
HYBRIDIZATION OF THYMIC CELLS

<table>
<thead>
<tr>
<th>Authors</th>
<th>Thymic Cell</th>
<th>Other Cell</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parkman et al., 1971</td>
<td>Human thymocyte</td>
<td>&quot;Mouse fibroblast&quot;</td>
<td>Cells produced &quot;human γ-globulin fragment&quot;</td>
</tr>
<tr>
<td>Parkman and Merler, 1973</td>
<td>Mouse thymocyte</td>
<td>clone 1D (L cell)</td>
<td>3 clones retain helper activity</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>no other thymic properties</td>
</tr>
<tr>
<td>Hyman and Kelleher, 1975</td>
<td>Mouse lymphoma</td>
<td>A9 (L cell)</td>
<td>All clones Thy-1 negative</td>
</tr>
<tr>
<td>Liang and Cohen, 1975</td>
<td>Mouse leukemia</td>
<td>LM(TK-) (L cell)</td>
<td>Clones Thy-1 negative but Tla positive</td>
</tr>
<tr>
<td>Namba and Waksman, 1975</td>
<td>Rat thymocytes</td>
<td>Mouse lymphoblast</td>
<td>Thymocyte antigen and MIF production</td>
</tr>
</tbody>
</table>
migration inhibitory factor. This experiment cannot be considered an intertypic fusion, since the mouse lymphoblastoid cells are closely related, developmentally, to thymocytes, but it does provide us with a control showing that thymus-related phenotypes can survive hybridization.

The studies of Parkman and his colleagues are most clearly relevant to my work, but are also most open to question. Parkman et al. (1971) state, in abstract form, that human thymocyte x mouse fibroblast hybrids produce a protein which can be precipitated with an antiserum to human $\gamma$-globulin. "Zero to 60% of colonies" were said to have been positive upon initial testing. Acrylamide electrophoretic analysis of the immunoprecipitate showed that the protein was neither whole $\gamma$-globulin, light chain, $F_{ab}$ fragment, or $F_c$ fragment. Since thymocytes themselves neither secrete $\gamma$-globulin nor give rise to cells that do, it seems likely that the protein produced by these hybrids, although cross-reactive with $\gamma$-globulin to some extent, may have had nothing to do whatever with the lymphoid system. No controls testing secretion of this protein by either parent were reported.

Parkman and Merler (1973) later reported, at greater length, the results of somatic cell fusions between mouse thymocytes (fractionated into classes of different buoyant densities) and cells of clone 1D, an L cell derivative. One hybrid clone was tested karyotypically, and was shown to have 80-95 chromosomes (mouse thymocytes have 40 acrocentric chromosomes, and clone 1D 50-55, of which about 20 are bi-armed). Three of the hybrid clones (it is not clear how many were tested, although 27 colonies were isolated) were said to have restored the ability of irradiated, bone-marrow-reconstituted mice to produce antibody-
secreting (plaque-forming) cells when challenged with sheep red blood cells. The hybrid cells did not display Thy-1 antigen, did not produce a graft-versus-host reaction when injected into suitably incompatible mice, did not form spontaneous rosettes with sheep red blood cells, and grew attached to plastic surfaces. Taken at face value, these results suggest that at least one lymphoid function, helper activity, can be retained in L cell-thymocyte hybrids.

The demonstration of helper activity in these hybrids, though difficult to explain away, is also quite surprising. For one thing, although thymic cells can give rise to T lymphocytes with helper activity, they cannot themselves contribute helper activity to irradiated hosts. It is surprising that these hybrids, while retaining one T cell property, seem to have lost the other morphologic, antigenic, and functional traits of their lymphoid parent. The finding that 3 hybrids (out of at most 27 tested) showed helper activity against sheep red blood cells raises other questions, because the proportion of T cells reactive against this antigen would be expected to be far lower than one in nine. None of the hybrids was found to provide helper activity against burro red blood cells, or to produce a graft-versus-host reaction in allogeneic mice, although these two antigenic stimuli would be thought to arouse at least as large a fraction of mouse T cells as would sheep erythrocytes. Perhaps sheep-red-cell reactive thymic cells are more susceptible to fusion than their burro- or alloantigen-responsive neighbors; but it is difficult to see why this should be so.

It is hard to abstract general conclusions from these five studies. The three studies in which Thy-1<sup>-</sup> cells were fused with Thy-1<sup>+</sup> partners resulted in Thy-1<sup>-</sup> hybrids. On the other hand, the work of Liang and
Cohen (1975) on Tla antigens and, given the above reservations, the work of Parkman and his co-workers, suggest that some of the characteristics of thymocytes can be expressed in L cell hybrids. The genetic controls on Tla expression are quite complex and not yet well understood (see Klein, 1975, pp. 241-251); furthermore, the behavior of the Tla antigens in the hybrids of Liang and Cohen (1975) differs from that characteristic of these antigens. Further work is clearly needed to clarify the fate of thymic phenotypes in thymocyte hybrids.

PART 1C: Hybridization of Friend erythroleukemic cells

Friend (erythro)leukemia cells (FLC) are near-diploid, tumorigenic, virus-transformed analogues of proerythroblasts. Many lines of "Friend cells" exist, some of independent origin, and some clonally related to others; it is not clear to what extent variations among the lines represent different stages of erythroid differentiation "trapped" by Friend virus transformation. Most Friend cell lines exhibit some erythroid properties (e.g. globin mRNA, erythroid antigens, enzymatic activities). The most dramatic property of Friend cells is the capacity of some lines, when treated with dimethylsulfoxide (DMSO), to progress to a normoblast-like stage. This maturation involves diminution in size, changes in enzyme activity, and a large increase in the amount of both globin mRNA and hemoglobin produced. The properties of Friend cells have been recently reviewed (Friend et al., 1974).

Quite a number of cell fusion experiments involving FLC have been reported. Orkin et al. (1975) described the properties of hybrids between two different FLC lines, one of which was better able than the
other to undergo DMSO-induced maturation. They found that the hybrids contained baseline (i.e. uninduced) levels of globin mRNA comparable to those of the parent cells, but that DMSO-treatment failed to bring about much increase in message content. Similar experiments, although with slightly different results, have been reported by Paul and Hickey (1974), and are not directly relevant to our discussion of intertypic fusion. These studies do show, however, that fusion itself need not abolish globin mRNA production, at least at the lower, baseline level, in FLC.

FLC have also been involved in several intertypic fusions, and these are summarized in Table 1-3. Fusion of FLC with 3 different clones of L cells, or with hepatoma cells, or with SV40-transformed human fibroblasts, all extinguish globin mRNA production, even if the hybrid cells are grown in medium supplemented with DMSO. On the other hand, fusion between FLC and human marrow cells produces hybrid lines in which globin mRNA (or globin itself) can be detected after DMSO treatment. One interpretation of these results holds that hybrids in which both parents are already committed to erythroid differentiation continue to produce globin, but that, for some reason, fusion with non-erythroid parents blocks production of globin mRNA. As suggested by the data in Table 1-3, however, (and shown in greater detail in the original papers), all those intertypic hybrids in which globin can be detected have lost the large majority of the chromosomes contributed by the non-erythroid parent. All intertypic hybrids in which globin expression is extinguished have retained large numbers of chromosomes from both parents. It would thus be very useful to examine a hybrid between FLCs and a non-erythroid cell whose chromosomes were lost.

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# Table 1-3

**Intertypic Friend Cell Hybrids**

<table>
<thead>
<tr>
<th>Authors</th>
<th>Other Cell</th>
<th>Chromosome Numbers</th>
<th>Globin Gene Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FLC</td>
<td>Other</td>
<td>Hybrids</td>
</tr>
<tr>
<td>Orkin et al., 1975</td>
<td>A9</td>
<td>40</td>
<td>50-60</td>
</tr>
<tr>
<td>Deisseroth et al., 1975a</td>
<td>B82</td>
<td>38-39</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>VA2 (SV40-transformed human fibroblast)</td>
<td>39</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>Human marrow (1°)</td>
<td>38</td>
<td>46</td>
</tr>
<tr>
<td>Deisseroth et al., 1975b</td>
<td>Human marrow (1°)</td>
<td>38</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2x 38</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>Chinese hamster fetal erythroblasts</td>
<td>38</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2x 38</td>
<td>22</td>
</tr>
<tr>
<td>Skoultchi et al., 1976</td>
<td>A9</td>
<td>39</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>LM(TK&lt;sup&gt;−&lt;/sup&gt;) (L cell)</td>
<td>37</td>
<td>45</td>
</tr>
<tr>
<td>Conscience et al., 1976</td>
<td>Hepa-la (hepatoma)</td>
<td>39</td>
<td>59</td>
</tr>
</tbody>
</table>

**Notes:** The hybrids tested by Deisseroth et al. (1975b) were uncloned populations; some cells had received a 2S Friend cell input. Note as well the segregation of human chromosomes in the primary human marrow fusions. Globin gene expression was tested by assay of either hemoglobin (for Chinese hamster) or globin mRNA.
early in the evolution of the hybrid clone.

All of the cells so far shown to extinguish FLC properties have not only been non-erythroid, but have been of types whose ancestors have passed through developmental branches leading away from the erythroid lineage. It would be interesting to examine the properties of FLC hybrids whose other parent, while not committed to red cell production, had not yet passed through such a branch point: e.g. hematocytoblast stem cells or one of the precursors of this cell type.

PART 1D: Teratocarcinoma as a model system for the mouse embryo

Nature has contrived serious methodological difficulties for the mammalian embryologist. Embryos are small, and are hidden away during the short period of time in which the developmental "action" takes place. Developmental biologists have recently begun to pay attention to mouse teratomas and teratocarcinomas as accessible models for embryonic systems, in the hopes that from these tumors they could derive useful amounts of developmentally-important materials, and could observe cells going through interesting transitions. Most research so far has been aimed at demonstrating the extent to which the model system imitates the natural one, but a few studies have produced insights into the surface molecules and viral susceptibilities of teratocarcinoma cells (and, by inference, of their natural counterparts) that would have been quite difficult to achieve without the tumor model.

The best review of the early work on mouse teratoma is that of Stevens (1967a). After his discovery (Stevens and Little, 1954) that mice of the inbred strain 129 were unusually susceptible to spontaneous
testicular teratoma, Stevens spent the next two decades studying the
go of these tumors. His influential studies (to be discussed
below) produced an apparent paradox whose recent resolution has clari-
fied the field considerably (see Damjanov and Solter, 1974, for a
review).

The first well-studied source of mouse teratomas was the genital
ridge of the male fetus. In ways that are not yet understood, male
primordial germ cells (PGCs) can give rise to embryonal carcinoma cells
(ECCs, the stem cells of teratocarcinomas; see below). This can occur
either spontaneously, in susceptible strains related to 129, or, in
certain other strains (e.g. A/He; Stevens, 1970a) when the genital
ridge containing the germ cell precursors is transplanted from the
fetus to adult testis. That primordial germ cells gave rise to the
tumor cells was suggested on circumstantial and morphologic evidence
by Stevens (1962) and by Pierce and Beals (1964), and confirmed by
Stevens (1967b), who showed that genital ridges homozygous for S1^J
(which, when homozygous, drastically reduces the number of PGCs in
the genital ridge) were incapable of giving rise to teratomas.

Other studies suggested, however, that teratomas could be produced
from embryonic cells at stages during which no PGCs were present.
Stevens (1968, 1970b) showed, for example, that when mouse embryos
from the two-cell to the 6th-day stage were implanted under the testis
capsule of an adult host, the implanted material produced growths which
included a variety of tissue types, sometimes including embryonal
carcinoma cells. Those tumors which contained a good proportion of the
ECCs were transplantable and were indistinguishable from the transplant-
able teratocarcinomas produced from genital-ridge-derived tumors.
Transplanted embryos could give rise to teratocarcinomas even though they were of strains (C3H) or sexes (female) whose PGCs were incapable of producing teratomas spontaneously or through genital ridge transplantation. Histological analysis suggested that the growths were derived from the transplanted embryonic cells themselves, rather than from PGCs to which the embryonic cells might hypothetically give rise.

Later work by Stevens and Varnum (1974) showed that ovarian teratomas were common in another inbred strain, LT, and that the tumors in these animals were derived from parthenogenetically-activated oocytes. Their careful histologic study showed that activated eggs underwent an initially normal development, but that disorganization usually began around the time of "blastocyst" formation, after which further growth gave rise to typical teratomas, and sometimes teratocarcinomas.

Teratocarcinomas, then, can be derived from primordial germ cells, from pluripotent embryonic cells in 3-6-day-old fetuses, and from activated oocytes in females. Damjanov and Solter (1974) have resolved these three apparently conflicting results into a convincing picture of teratoma development whose central tenet is that teratomas develop whenever "mesectodermal precursor cells" (MPCs) are placed into a particular kind of "wrong" environment (wrong, that is, for normal embryogenesis). They argue that those cells in the egg cylinder which ordinarily give rise to the ectodermal and mesodermal tissues of the embryo proper can, in a suitable extra-uterine environment, give rise to teratomas at very high frequency. In their view, embryonal carcinoma cells are (at least early in their history) identical to the mesectodermal precursor cells except that they are misplaced.
These precursor cells are obviously present in normal 6-day mouse embryos, and can be produced through normal developmental events from 3-day embryos. Stevens and Varnum (1974) have shown that activated LT eggs progress through a series of developmental stages quite similar to the changes which take place in a normal embryo, sometimes reaching the primitive-streak stage (7.5 days of gestation), but that disorganization eventually sets in followed by teratoma formation. Therefore these parthenotes, too, contain misplaced mesectodermal precursor cells.

The mechanism by which male primordial germ cells give rise to teratoma stem cells is less clear. Stevens, in his examinations of thousands of early genital-ridge-derived spontaneous and induced teratomas, has never seen stages corresponding to mouse cleavage embryos; the first recognizable teratoma cells resemble those of the blastocyst inner cell mass (Stevens, 1975). Without further investigation into the activation process in male PGCs, all one can say is that such a transition does occur, and that the mesectodermal precursor cells thus produced, in the absence of their "natural" environmental signals, give rise to growths which are both disorganized and progressively growing: i.e., teratomas.

Damjanov and Solter (1974) argue, then, that ECCs (and hence teratomas) can be produced in any of these three ways, that the cells produced by the three methods are indistinguishable, and, most radically, that embryonal carcinoma cells are in no way different from the mesectodermal precursor cells from which they come.

This last idea has profound implications, and may well be true. Most tumors are likely to have been derived from cells "transformed" by
chemical, physical, or viral agents. These tumor cells may still bear stigmata of the transforming agent (viral nucleic acid sequences, or, hypothetically, mutations in genes important for growth control), and their loss of growth control seems to be irreversible. If ECCs, however, are normal although misplaced, the factors responsible for their tumorigenicity might be very different from those which effect most other cell types. ECCs, for example, characteristically retain near-diploid chromosome numbers (Guénet, 1974), whereas most other tumors exhibit more severe aneuploidy. It is tempting to view these differences in karyotype as effects of underlying differences in the ways in which these cells have been rendered tumorigenic.

One important implication of the "normal-but-lost" hypothesis is that an ECC placed "back" into a blastocyst, in which it will be exposed to the environment appropriate for normal MPCs, will be able to regain both growth control and a capacity for normally organized and properly timed differentiation. The recent exciting work of Brinster (1974), Papageorgiou et al. (1975) and Mintz and Illmensee (1975) suggests that ECCs can indeed act just as dermal precursor cells if inserted into the blastocyst cavity of normal mouse embryos.

PART 1E: In vitro systems for the study of teratocarcinoma

Tumor cells are often studied in tissue culture for the insights one can derive into their untransformed analogues, and the preceding discussion suggests that ECCs may be unusually good models for the pluripotent mesectodermal cells of the early mouse embryo. Further tests of this assumption will be presented in Part 1F, below. I want
now to discuss the recent work on in vitro culture of teratocarcinoma-derived cells, which has made possible experiments not easily carried out on the tumors themselves. Gail Martin's recent review (Martin, 1975) deals largely with these cultures and is essential reading for newcomers to this field.

Figure 1-1 shows the relationships among the different kinds of teratocarcinoma-derived cell cultures and the tumor forms from which they come. Teratocarcinomas, whether embryo-derived, spontaneous (testicular or ovarian), or produced by genital-ridge transplantation, are usually maintained by repeated subcutaneous passage in histocompatible mice. Some tumors have also been converted into an unusual ascitic form, in which clusters of cells, called "embryoid bodies" (Stevens, 1959; Pierce and Dixon, 1959), usually containing a central core of ECCs surrounded by primary endoderm cells together with varying amounts of other cell types, grow free in the peritoneal fluid. Embryoid bodies are maintained by injection of infected ascitic fluid into successive host animals. Embryoid bodies can also produce solid growths, with the usual chaotic pattern of embryonal carcinoma and differentiated tissues, either after implantation on the peritoneal wall after intraperitoneal inoculation, or if a suspension of embryoid bodies is injected subcutaneously.

Cell cultures can be initiated from either subcutaneous tumors or embryoid bodies. These primary cultures contain varying proportions of ECCs and differentiated elements, among which primary endoderm is usually predominant at first. By the time a primary culture is sufficiently well established to permit subculture, much cell death has occurred, possibly involving selection for those cells best adapted to growth.
Figure 1-1

Derivations of teratocarcinoma cell lines and tumors.
LT oocytes, male genital ridge, transplant embryo

1° teratoma

teratocarcinoma

solid form

Embryoid bodies

mixed 1° cultures

ECC à la Martin

much selection

mixed 2° cultures

impure ECC

e.g. SIKR

monomorphic lines

e.g. keratinocyte

"pure" ECC (pluripotent)
e.g. PCC4

fibroblast

"Nullipotent" e.g. F9
in vitro. These initial populations can be subcultured repeatedly as mixtures composed of ECCs, their newly-differentiated derivatives, and the mitotic progeny of those differentiated cells which can still divide. The work of Lehman et al. (1974), Gearhart and Mintz (1974), Levine et al. (1974) and Teresky et al. (1974) has shown that these primary and secondary cultures can be used to follow morphological and biochemical differentiation of teratocarcinoma cells in culture.

There are a number of ways in which less heterogeneous cell lines can be derived from such a mixed culture. The earliest reports of teratoma-derived cultures (Finch and Ephrussi, 1967; Rosenthal et al., 1970; Kahan and Ephrussi, 1970) showed that cloning of cells from mixed cultures failed to abolish pluripotency. Cloned cells were able to give rise not only to multi-differentiated tumors, but also to morphologically heterogeneous culture populations. By subculturing from mixed populations in such a way as to favor transfer of the ECCs, which attach only loosely to plastic surfaces, one can maintain a population which is made up predominantly of pluripotent cells, but in which contamination with freshly differentiating cell types repeatedly takes place (e.g. Lehman et al., 1974). The SIKR line used by Jami et al. (1973) for cell fusion experiments has been shown to be a "mixed clone" of this sort (Martin and Evans, 1974).

Teratocarcinoma-derived cell lines in which pluripotent cells are no longer present also exist. Many of these seem to consist of fibroblastoid cells not easy to classify further. More interesting "monopotent" lines have, however, been produced. Boon et al. (1974) have derived two lines of myoblastoid cells by mechanical isolation of beating foci found in a mixed culture. Rheinwald and Green (1975)
isolated a line of keratinocytes from a mixed culture in two stages: first, the mixed population was propagated under conditions which favored the growth of epithelial cells, after which the population was cloned and a well-differentiated colony picked for further study. Mixed cultures can also convert to nonpluripotent cultures simply by overgrowth of a differentiated cell type which divides more rapidly than the ECCs under the culture conditions used.

Several laboratories have been able to isolate cell lines which seem, at least on morphologic grounds, to consist only of embryonal carcinoma cells. Jakob et al. (1973) have, for example, isolated several of these lines, including a clone called PCC4azal, the azaguanine-resistant derivative of clone PCC4 which was used in the experiments I have carried out and will discuss below. It is not clear why the lines isolated by this group show so little tendency to differentiate in logarithmic cultures, since Jakob et al. (1973) used the same techniques (cloning and selective subculture) which in other hands produced "mixed clones" partially contaminated with freshly differentiating cell types. These "pure" ECC lines usually retain the ability to produce multi-differentiated tumors, but only some of them (Nicolas et al., 1975) are able to differentiate in tissue culture, and then only under particular culture conditions. PCC4azal, the cell line used in my work, is able to differentiate in vivo (Jakob et al., 1973) and after special manipulations in vitro (Sherman, 1975a), but appears to consist entirely of ECCs under routine culture conditions.

Some ECC lines, after many months of growth in vitro, are found to have lost the ability to differentiate in tumors, although they
resemble the pluripotent ECC lines in their other characteristics. These cell lines are said to be "nullipotent". F9 is the best studied line of this type; it was isolated by Grandchamp and Ephrussi but first mentioned in Bernstine et al. (1973). Recent work (Sherman and Miller, in preparation) suggests that F9 cells are able to produce parietal yolk sac endoderm, under special growth conditions in vitro, and as small, scattered nodules in tumors, but seem to be unable to generate other kinds of differentiated cells.

Martin and Evans (1975a, b, c) have recently developed a method for isolating pure lines of ECCs which seems likely to supplant the other methods used in earlier work. Their procedure involves the mechanical removal, from primary or secondary mixed cultures, of single embryonal carcinoma cells under the dissecting microscope. These isolated cells are then cloned on fibroblast monolayers. Martin and Evans postulate (1975a) that unselected ECCs require some product of the "feeder" fibroblasts. In their view, previous attempts to isolate pure ECC lines without the use of feeder layers may have failed because of selection for ECCs which continually gave off the fibroblastoid derivatives they needed to provide a feeder monolayer. The success of the Paris group (Jakob et al., 1973) in isolating apparently pure ECC lines without feeders may have been due to growth conditions which selected for feeder-independent cells; it is not clear to what extent this selection may have modified the other properties of the pluripotent Parisian lines. Using their single cell cloning technique, Martin and Evans have repeatedly obtained, at about 40% cloning efficiency, lines of ECCs which, when kept on feeder layers, show little tendency to produce differentiated offspring. This group
has also defined conditions (Martin and Evans, 1975a, b, c) under which their cell lines can be induced to differentiate. Under some regimens, differentiation begins with the formation, in culture, of structures much like embryoid bodies. This system seems to furnish an excellent in vitro model for two of the earliest determinative transitions of the mouse embryo: formation of primary endoderm from the "embryoblast" cells of the inner cell mass, and the (endoderm-induced?) formation of mesodermal derivatives from the remaining MPCs. The fidelity with which the differentiating cultures of Martin and Evans mimic the early stages of ontogeny reinforces our conviction that ECCs are good models of embryonic mesectodermal precursor cells.

We will test this faith further in the next section, devoted to the properties of the embryonal carcinoma cell.

PART IF: Properties of embryonal carcinoma cells

My aim in discussing the properties of embryonal carcinoma cells is twofold: both to show by what tests these cells can be distinguished from other kinds (e.g. other parents in a hybridization experiment, or other cell types in a mixed culture) and to test further the claim that ECCs resemble closely the mesectodermal precursor cells in the normal mouse embryo.

Developmental properties.

The important developmental properties of ECCs are two: these cells are pluripotent, and they are tumorigenic. The pluripotency of
individual (i.e. cloned) ECCs has been demonstrated in vivo by Kleinsmith and Pierce (1964), and in vitro by Finch and Ephrussi (1967) and by Rosenthal et al. (1970). ECCs are thought to confer transplantability on teratocarcinomas (see Damjanov and Solter, 1974, for a discussion), because the capacity of a tumor to survive transplantation seems to be dependent on the presence of morphologically recognizable ECCs within it. Although Kleinsmith and Pierce (1964) were able to obtain tumors at a low rate (43 tumors from 1790 animals inoculated) by injection of single ECCs, doses of $10^4$ cells/animal are usually necessary to produce tumors in most of the injected mice (Jakob et al., 1973). Boon et al. (1975) have described the production of non-tumorigenic lines from mutagenized ECC cultures.

MPCs are, of course, also pluripotent. The work of Solter and Damjanov (1973) suggests that for a transplanted embryo to give rise to a tumor, it is necessary that the MPC-containing half of the egg cylinder be included in the graft. Over 50% of transplanted embryos, earlier than 8 days of gestation, produce teratomas, of which about half contain ECCs and are thus likely to be teratocarcinomas (Stevens, 1970b; Solter et al., 1975). This high rate of tumor formation suggests that at least some of the MPCs in the 2-7-day-old mouse embryo are capable of producing transplantable tumors if placed into the right environment, even in the absence of carcinogenic treatment.

The discovery of Sobis and Vandeputte (1975) that benign teratomas could be produced in rats by displacement of visceral yolk sac endoderm to the outside of the uterus (with fetectomy) suggests that MPCs may not be the only pluripotent cells in the early post-implantation embryo. These rat tumors, however, contain no embryonal carcinoma cells and are
not tumorigenic; they therefore furnish no evidence against the claim that MPCs are the cells which give rise to ECCs in the mouse systems under discussion.

Morphological properties.

ECCs, however derived, have a characteristic ultrastructure; the most recent review is that of Damjanov and Solter (1975). In tumors and in culture, these cells typically exhibit the following features: a high ratio of nuclear volume to cytoplasmic volume (compared to most well-differentiated cells); a prominent, usually single nucleolus, centrally located in a nucleus with little other condensed chromatin; and a cytoplasm which contains many free ribosomes but few other organelles—mitochondria are present but scarce. The ultrastructure of these cells is very similar to that of both primordial germ cells in the male (Pierce and Beals, 1964) and mesectodermal precursor cells (Damjanov et al., 1971a). Taken together, these structural elements combine to produce a phase contrast image (see below) of these cells which one can, with a little experience, learn to recognize easily, and which is easy to distinguish from that of most other cell lines. Cells cultured from the inner cell mass of mouse blastocysts strikingly resemble teratocarcinoma-derived ECCs (see figure 1 in Sherman, 1975b, for a photographic comparison). Paulin et al. (1976) have described ECC-specific patterns of non-histone chromatin protein and soluble nuclear protein which may contribute to the unusual nuclear structure (not to say developmental properties!) of these cells.

ECCs in culture also exhibit a characteristic growth habit: upon
subculture, even from a single-cell suspension, they tend to attach in clumps, from which the individual cells show little tendency to migrate. ECCs in some lines grow as mounds of cells more firmly attached to one another than to the substrate. This colonial behavior in culture may be a reflection of the tendency of ECCs in tumors to form islands of cells surrounded by streams of differentiated cell types.

Alkaline phosphatase.

Alkaline phosphatase (APase; E.C. 3.1.3.1), an enzyme demonstrable by its hydrolysis of phosphate esters at alkaline pH, but whose role in cellular metabolism is not understood, is present in large amounts in a number of somatic tissues (bone, intestine, kidney, liver, placenta, white blood cells; see Kaplan, 1972, for a useful review). Although APase is not present in high levels in most cultured cell lines or in most cell types in the early embryo, high specific activities can be demonstrated in ECCs both in tumors (Damjanov et al., 1971b) and in culture (Bernstine et al., 1973). The demonstration that MFCs, alone among the cells of the mouse egg cylinder, have high APase levels (Solter et al., 1973) supports the contention that these cells are the normal counterparts of the ECC. APase is also present in high specific activity in primordial germ cells (Chiquoine, 1954). Those non-pluripotent cell lines derived from teratocarcinoma which have been tested for APase, like most differentiated tissues in teratocarcinoma tumors, have proven to have little activity (Bernstine et al., 1973). Thus, this enzyme serves as a useful marker for the ECC, and its loss, a useful though by no means infallible marker for ECC differentiation.
Response to viruses.

Lehman and his co-workers have tested the response of cultured ECCs to several mammalian viruses, in the hopes that if differences could be found between ECCs and other cultured cells in their response to virus infection, these differences might provide insights into viral metabolism and into the pluripotent state. Swartzendruber and Lehman (1975) showed that SV40, although able to bring about T-antigen synthesis in abortively-infected non-pluripotent mouse cells, was unable to produce this antigen in ECCs. Attempts to transform ECCs with SV40 met with failure. Differentiated cells present in mixed cultures, however, were able to make both T and V antigens, and were found to be susceptible to SV40 transformation. Similar results were obtained with another oncogenic DNA virus, polyoma; only the non-pluripotent cells were able to produce polyoma T and V antigens, and made infectious virus as well. (Most mouse cells are permissive for polyoma, but support only abortive infection, with occasional transformation, by SV40.) In a second paper, Lehman et al. (1975) demonstrated that ECCs were also resistant to infectious SV40 DNA, suggesting that resistance is dependent on some event subsequent to viral attachment and unpacking. Lehman et al. (1975) also showed that an unrelated RNA virus, mengo-virus, is able to replicate in ECCs, but that virus yield is much reduced. Jaenisch and Mintz (1974) have shown that exposure of pre-implantation mouse embryos to SV40 virus can lead to the production of adult mice whose cell nuclei contain SV40 nucleic acid sequences, but one cannot deduce from their data the cell type or embryonic stage at which the viral information was introduced.
Miller et al. (1976; submitted for publication) have shown that ECCs are resistant to infection by a mouse parvovirus, MVM; some of these results will be presented below.

The resistance of ECCs to a number of viruses which do infect non-pluripotent mouse cell lines is relevant here largely as a way to distinguish ECCs from other cell types. It seems likely, though, that further study of these phenomena will give us insights into viral metabolism, and into changes in the intracellular milieu during early embryogenesis.

Surface antigens.

Studies of the surface antigens of ECCs have provided, in addition to useful markers for these pluripotent cells, new perspectives on the genetic control of surface molecules and the role of these molecules in ontogeny.

The initial paper in a series of influential collaborative reports was that of Artzt et al. (1973). These workers showed that when 129/Sv mice were inoculated with cells of a syngeneic "nullipotent" embryonal carcinoma cell line, F9, they produced hyperimmune serum cytotoxic for F9 cells. A series of direct cytotoxic tests and absorptions showed that the sera detected antigens present on all tested ECC lines, but not present on any of the many non-pluripotent lines tested, including some also derived from 129/Sv teratocarcinomas. These "F9" antigens were not found on thymus cells, but were detected on spermatozoa and cells in testis suspensions. Immunoperoxidase labelling showed that cross-reacting (and possibly identical) antigens were present on
cleavage-stage mouse embryos.

By using syngeneic cells for immunization, Artzt and her collaborators hoped to have manufactured a serum which detected only those surface antigens which, though present in early embryos and their teratoma-derived analogues, had disappeared from the embryo by the time self-tolerance was initiated. (It is not surprising that sperm might prove to be an exception, since male germ cells are inaccessible to cells of the immune system and can give rise to potent autoantibodies if inoculated around this "blood-testis barrier." ) The demonstration of antigens common to embryonal carcinoma cells and early mouse embryos supported the growing conviction that these cell lines were good models for early embryonic cells.

In a second paper, Artzt et al. (1974) showed that spermatozoa could absorb anti-F9 cytotoxic activity (as measured with F9 target cells) to a degree that depended on their genotype. Sperm from an animal which was heterozygous for the mutant allele \( t^{12} \) (at the complex T/t locus) were only half as effective in removing anti-F9 cytotoxic activity as sperm from an animal homozygous for the wild-type allele, \( +t^{12} \). They concluded that the F9 antigen was probably specified by the wild-type allele.

The T/t complex comprises a series of interacting loci with effects on early embryonic morphogenesis, sperm structure and function, and recombination in the chromosome segment stretching at least from the T locus to the H-2 complex 13.5 centimorgans distal on chromosome 17. Three useful reviews (Gluecksohn-Waelsch and Erickson, 1970; Bennett, 1975; and Klein, 1975, pp. 251-274) are available to acquaint the novice with the large amount of work which has been done on this
most mysterious and fascinating of mammalian complex loci. The work of Bennett et al. (1972) had suggested that the effects of T/t locus mutants on spermatozoa might be mediated by surface molecules. The work of Artzt et al. (1974) just discussed showed that the effects of these genes on embryonic development might also involve surface antigens, and that these molecules could be studied using the teratocarcinoma model systems. There are several complementation groups of recessive-lethal T/t locus mutants; members of a particular group block embryogenesis at a group-specific stage. The group including t^{12} (the wild-type allele of which had been associated with F9 antigen) blocks embryogenesis at a very early stage: homozygous t^{12}/t^{12} embryos are unable to proceed from morula to blastocyst. The demonstration that F9 antigen was present on late cleavage stage embryos was seen by Artzt et al. (1974) as consistent with the idea that this surface molecule is necessary for the transition to blastocyst, and consistent with the notion that ECCs are analogous to morula cells.

A third paper (Vitetta et al., 1975) showed that F9 antigen, isolated from other surface molecules by immunoprecipitation, shared certain biochemical properties with H-2 antigens. Artzt and Jacob (1974) showed, around the same time, that F9 cells lacked H-2 antigen, at least insofar as could be demonstrated by cytotoxic absorption tests. They suggested that the F9 antigen might take the place of H-2 on early embryonic cells and ECCs.

The idea had been growing for some time that the association, on chromosome 17, of these two most fascinating and mysterious of mammalian complex loci had better be explained. (The current basis for this suspicion is reviewed in Artzt and Bennett, 1975.)
linkage disequilibrium discovered by Hammerberg and Klein (1975) between the two complexes further suggests that the similarities and linkage between these sets of loci may be more than just a historical accident. Biochemical studies of the F9 antigen are still in their preliminary stages. The smaller of the F9 polypeptides is not immunologically cross-reactive with mouse β-2-microglobulin, its counterpart in the H-2 molecule (F. Jacob, personal communication). Work to compare the amino acid sequence of the F9 and H-2 molecules is in progress. The identity of the H-2-associated molecule(s) which interact with products of the T/t locus, and the nature and value of this interaction are still entirely unknown.

These studies lead to a neat model of surface antigen progression in developing embryos, in which F9 (+t12) antigen appears before the morula stage, somehow assists in the morula-to-blastocyst transition, and is eventually replaced by H-2 molecules. The products of the other T/t loci might well follow similar schedules at appropriate times and in appropriate cell types. There are, however, a number of findings which, while not inconsistent with this model, suggest that the model may be too simple. For one thing, F9 antigen is present in mesectodermal precursor cells in mouse embryos at least as late as the egg cylinder stage (L. Stevens and F. Jacob, personal communication), as well as on ECCs derived from embryos more mature than blastocysts. Similarly, H-2 antigen is thought not to appear in the mouse embryo until 7 days after fertilization (see Billington and Jenkinson, 1975). It is clear, then, that the postulated replacement of F9 antigen by H-2 does not occur until long after the stage at which the +t12 gene is known to act (the morula-to-blastocyst transition occurs on the 4th
day after fertilization.) Furthermore, although no one has yet found an ECC which lacks F9 antigen, there are several non-pluripotent lines which express antigens which can be recognized by anti-F9 serum in immunofluorescent assays (my unpublished results and Artzt, personal communication.) Furthermore, a similar anti-ECC serum produced by Stern et al. (1975) can be absorbed successfully, not only by ECCs, but by mouse brain and kidney as well. Although this 129-anti-SIKR serum was produced by a protocol similar to that used for anti-F9 serum, and has a similar spectrum of reactivity (including ECCs, sperm, and morulae), it is not clear whether the product is recognizes is the "F9" antigen, or is even specified by a gene at the T/t complex. Stern et al. (1975) characterized their serum by immunofluorescent assays, whereas Artzt and her colleagues use cytotoxic tests; it is not clear whether the sets of teratocarcinoma-specific antigens detected by these two methods are identical.

Stern et al. (1975) also looked for the presence of H-2 and Thy-1 antigens on ECCs and their differentiated progeny. They confirmed the result of Artzt and Jacob (1974), mentioned above, that ECCs lack H-2, and found that Thy-1 antigen was also undetectable, but that both antigens were present in ECC-derived cultures containing large numbers of differentiated cells. The earliest of the differentiated cell types produced in their system (primary endoderm) was, however, devoid of all three antigens: H-2, Thy-1, and the ECC antigen detected by their anti-SIKR serum.

To sum up: embryonal carcinoma cells seem to lack the H-2 antigens found on most other cell types, and to possess their own surface antigens missing on most cells of later embryonic and adult stages. A
similar antigenic repertoire is characteristic of mouse morulae, and possibly of the mesectodermal precursor cells of the egg cylinder as well.

PART 1G: Somatic cell hybridization of teratocarcinomas

Besides the work to be described in this thesis, there have been five reports, two of them preliminary, of hybridization between cultured teratocarcinomas and other, non-pluripotent cell types.

In the earliest of these, Finch and Ephrussi (1967) fused a line of feeder-cloned, 402 AIII embryoid-body-derived cells, shown to be able to produce multi-differentiated tumors, with either of two mouse fibrosarcomas: LM(TK⁻) clone 1D, or 2472-6-3, a clone of Sanford's "high cancer" line NCTC 2472. The teratoma-derived parent, called clone 8, was said to yield cultures "homogeneous in appearance," but the published photograph shows, at the top, cells which seem intermediate in morphology between ECCs (which make up most of the colony shown) and the epithelial cell types which often appear in "mixed clone" cultures. The fibroblast cells each carried an enzyme deficiency preventing growth in the selective HAT medium, but the teratoma line carried no selectable marker. Hybrids were produced by mixing the two parental cell types without addition of Sendai virus. HAT medium was used to kill the fibroblast parent, and colonies of putative hybrids were picked for further analysis because they were "distinguishable from teratoma cells morphologically and by their higher growth rate."

Each of the three parents used in these experiments carried at least one marker chromosome not found in the other cells, and karyotypic
analysis showed that the picked colonies were in fact made up of hybrid cells. Seven of the hybrid clones were shown to produce tumors indistinguishable histologically from the fibrosarcomas formed after inoculation of LM(TK⁻) or 2472-6-3. There was no evidence of either embryonal carcinoma cells or differentiated elements (besides fibroblasts) in the hybrid tumors.

A very similar study has been reported by Jami et al. (1973). This group produced and studied hybrids between LM(TK⁻) clone 1D and a "mixed clone" teratocarcinoma SIKR (Evans, 1972; Martin and Evans, 1974). Hybrids were produced with the help of Sendai virus, the fibroblast parent killed with HAT, and "hybrid colonies...identified by their cell morphology which is distinct from SIKR colonies."

Karyologic analysis showed that the two hybrids isolated had more than 98% of the "expected" chromosome number for a 1:1 fusion product between the two parents. The hybrid cells formed tumors in (129 x C3H)F₁ mice but not in 129 or C3H animals, suggesting that they retained at least some of the histocompatibility genes of both parental cells. Isozyme analysis showed that in those instances where the two parental cells differed in their proportions of a particular isozymic form, the hybrid pattern resembled that of LM(TK⁻). In particular, "two faster molecular forms of lactate-dehydrogenases were found in SIKR" (presumably LDH A₁B₁ and A₂B₂; see below) and were absent both in clone 1D and in the hybrids. The hybrids formed tumors in F₁ mice which, like those of the fibroblast parent, contained only fibrosarcomatous tissue, devoid of either embryonal carcinoma or any of the variety of differentiated cell types found in SIKR tumors. When cell lines were initiated from some of the tumors, 4 of the 5 lines derived...
were found to have retained more than 94% of the chromosome number characteristic of the inoculated cells; the other tumor had lost 25% of the original number.

A report by McBurney (1976), which deals largely with the isolation and characterization of several ECC lines, mentions hybridizations between two of these lines (OC15 and OC49) and cells of the established, subtetraploid, non-tumorigenic mouse fibroblast line, 3T3. A ouabain-resistant, TK- variant of 3T3 was used, and ouabain employed to select against the ECC lines. The 3T3 cells were killed in HAT. All the hybrid colonies were fibroblastic in morphology and, like their 3T3 parent, contact-inhibited for growth. OC49 is a near-tetraploid ECC line, and contains more chromosomes than the 3T3 line used; 3T3 x OC49 hybrids were also fibroblastoid. The hybrid cells were not able to form tumors (probably because the 3T3 parents originated in an outbred strain of mice) but, when cultured under conditions which promote differentiation of the ECC parental cells, gave rise only to fibroblastoid cells. McBurney (1976) has also shown that forcing ECCs to become tetraploid by treatment with cytochalasin B (a drug which permits nuclear division but blocks cytokinesis) does not entail a loss of pluripotency. Loss of pluripotency in some kinds of teratocarcinoma hybrid cells cannot therefore be attributed simply to increased chromosome number.

If taken at face value, these results suggest that hybrids between a pluripotent cell, such as those which predominate in the teratocarcinoma cultures used for the fusions, and a differentiated fibroblast cell can resemble the non-pluripotent parent. A more detailed criticism of this conclusion will be reserved for later.
Two further (and preliminary) accounts of fusions involving teratocarcinoma cell lines have appeared in print. Evans (1975) mentions the unpublished thesis research of his student, K. Sit, who produced two series of hybrids between SIKR and differentiated parents. Two clones of hybrids between SIKR and A9 (a mouse fibroblast closely related to LM(TK⁻)) are said to have produced only fibrosarcomatous tumors, in agreement with the results mentioned above. A series of 8 hybrid clones between SIKR and N86, a mouse neuroblastoma derived from the A/J Cl300 tumor, are said also to have given rise to fibrosarcomatous tumors without evidence of neural tissue. In culture, however, these hybrids are said to have had a high content of monoamines, which are characteristic of the neuroblastoma parent, but not of SIKR. Both series of hybrids were apparently isolated without biochemical selection against the SIKR parent; i.e., hybrid colonies may have been chosen for study on morphological criteria.

A control series of "self-hybrids" (SIKR x SIKR) was created by mixing SIKR cells with Sendai virus and picking "colonies of polyploid cells, which could be recognized by their greater size." These putative hybrids, with modal chromosome numbers ranging from 71 to 143 (SIKR mode is 41), were said to produce fibrosarcomas. Evans suggests that this surprising result could have come about in one of two ways: a) fusion itself might be able to bring about restrictions of the hybrids' developmental potential, or b) the fibroblastic cells known to contaminate SIKR cultures might be especially susceptible to Sendai-mediated fusion.

Another series of teratocarcinoma hybrids has been constructed by Bernstine and Ephrussi (1975) and reported in preliminary form. These
cell lines are the product of fusion between a neuroblastoma line (N18TG2, a close relative of the line used by Sit and Evans) and H3.6, described only as an "embryonal carcinoma line" with high alkaline phosphatase activity. The emphasis in this report is on the APase activities in the hybrids. The ECC parent has 18-fold more activity than the neuroblastoma parent, but hybrids have from 1.1- to 7.6-fold more APase than even the ECC parent. A portion of this increase may be due to changes in the response to growth inhibition in crowded cultures, in that Bernstine and his co-workers have shown that APase can increase at confluence as much as two-fold in ECCs. It will be important to learn whether the APase activity in the hybrids is like that of the ECC or of the neuroblastoma, since related clones of N18 neuroblastoma cells have very high APase activity (my unpublished results). Bernstine's preliminary results using heat denaturation kinetics suggest that the enzyme in the hybrids resembles that of the ECC parent, but more work is needed.

The report of Bernstine and Ephrussi (1975) contains very little information about what their hybrid cells look like. One clone, NT3, is said to make up a "homogeneous population of fibroblastic cells." The other two primary clones, NT1 and NT2, are said to exhibit morphological heterogeneity in vitro. Subclones of homogeneous type have been derived from the more heterogeneous primary clones. Tumors derived from NT1 and NT2, and from their subclones, are said to have been composed of "one predominant cell type" without "organized structure"; "differences between tumors produced by various clones have been noted." One cannot tell from these hints whether these hybrid cultures and tumors contained ECCs, neural elements, fibroblasts,
or perhaps other kinds of differentiated cells.

Jami and Loeb (1975) have mentioned unpublished work of Jami and Diacumakos to the effect that neuroblastoma x teratoma hybrids resemble neuroblastomas. No details are given.

In summary, then: there is as yet no good evidence that teratocarcinoma-derived hybrid cells can retain pluripotency, although the hybrid lines of Bernstine and Ephrussi (1975) may, when reported in more detail, retain some of the properties of ECCs. Three sets of hybrids between teratocarcinoma lines and mouse fibroblasts have resembled their fibroblast parents, although it is difficult in some cases to know whether the hybrid actually involved an embryonal carcinoma cell. In all these studies, except the case of McBurney's (1976) 0049 x 3T3 hybrid, the non-teratoma parent initially contributed more chromosomes to the hybrid cells than the teratoma-derived parent, so that controls to study the effect of more nearly equal gene dosage would be useful.

PART 1H: Production of α-fetoprotein in teratomas

α-fetoprotein (AFP) is a glycoprotein of molecular weight 68,000 found in high concentrations in amniotic fluid and the serum of third trimester mammalian embryos, but which is not present in the sera of normal (nongravid) adults. Certain differentiated tumors secrete AFP, most notably hepatomas and teratomas, so that detection of this substance has become a useful tool in the management of patients with these diseases. Abelev (1974) has written a fine review of AFP as a marker of embryo-specific differentiation in tumors and in normal
embryos. A minor controversy has arisen over the question of which cells in a teratocarcinoma produce AFP, and it is the purpose of this brief section to come down on one side of the controversy.

In the normal embryo, AFP synthesis occurs in the yolk sac endoderm, and later in the liver (Gitlin and Boesman, 1967; Gitlin et al., 1972). Using an immunofluorescent technique, Engelhardt et al. (1973) showed convincingly that AFP in mouse teratocarcinomas was, not surprisingly, associated almost entirely with endodermal derivatives: visceral yolk sac endoderm, and epithelia lining glands and tubules. Further supportive evidence for the proposition that AFP is a product of differentiated endodermal cells is summarized by Abelev (1974). Embryonal carcinoma was present in the tumors analyzed by Engelhardt et al. (1973), and was found not to be associated with AFP-specific fluorescence.

The work of Kahan and Levine (1971), however, had suggested that ECCs did produce AFP. Their conclusion was based on two pieces of evidence. In the first place, they found a "serum α-1-protein" in mice carrying transplantable teratocarcinomas (which contain ECC), but not in those with "well-differentiated" primary teratomas (which do not). The amount of endoderm in these tumors was not, however, given. More significantly, a cloned cell line, T17 8-10, consisting in vitro of a "compact monolayer of undifferentiated epithelial cells", and which gave rise to tumors consisting almost entirely of embryonal carcinoma with sparse parietal yolk sac, was shown to produce this α-1 protein, in vitro and in tumors.

On the whole, the evidence strongly supports the idea that AFP in teratocarcinomas originates in endodermal cells. If the protein
studied by Kahan and Ephrussi was in fact AFP, its production by the poorly-differentiating line \( T_{17} \) 8-10 is surprising. It is possible that this line might not be an ECC line at all, but rather a poorly differentiated parietal yolk sac carcinoma (no photographs are shown); another possibility is that \( T_{17} \) 8-10 represents a rare variety of ECC which has acquired the capacity to produce AFP. The immunohistological studies of Engelhardt et al. (1973) show clearly that when embryonal carcinoma and visceral yolk sac endoderm are both present in a teratocarcinoma, only the latter contains detectable AFP. My work (to be reported below) is quite consistent with this conclusion.

PART II: Experimental strategy.

The work reported in this dissertation concerns the production and characterization of two series of somatic cell hybrids. The PCT hybrids are the products of fusion between cells of a pluripotent embryonal carcinoma line, PCC4azal, and normal mouse thymocytes. The PCF hybrids result from fusions between PCC4azal and a line, FBU, of Friend erythroblastic leukemia cells. The purpose of this section is to show that these particular hybrid series could help to clarify some of the issues discussed above.

Nearly all the intertypic hybrids previously studied have involved non-pluripotent, "differentiated" parental lines, whose cells contain molecular machinery responsible for the maintenance of a particular epigenetic state throughout the cell cycle and through mitosis. The eventual phenotype of the hybrid cells, therefore, reflects the conflicts and compatibilities between two different sets of stabilizing
influences. ECCs, though phenotypically stable under some conditions, must contain a molecular apparatus, not present in most other cells, which permits the ECCs to generate the entire diverse repertoire of stable, "mature" epigenetic states.

It is not easy to predict the phenotype of a fusion product, one (and only one) of whose parents is able to produce, through normal developmental events, cells much like those of the other parental line. One might argue that (e.g.) the PCF hybrids ought to resemble the differentiated Friend cell parent, since a) the Friend cell is known to produce molecules which can impose an erythroid "set" on newly synthesized mouse haploid genomes; and b) PCC4azal is known to contain a genome in such a state that it can, under conditions that occur in normal development, adopt such an erythroid "set". The findings of Finch and Ephrussi (1967) and of Jami et al. (1973), which showed that teratoma-fibroblast hybrids are fibroblastoid, support this prediction.

On the other hand, the "signals" which Friend cells employ to insure the erythroid nature of their mitotic progeny are very likely to be distinct from those to which erythroid-bound pluripotent genomes are exposed. Pluripotent cells must ordinarily progress through a number of stages, starting with the hematocytoblast or its earliest precursor, before giving rise to the basophilic erythroblasts which the Friend cells (Conscience et al., submitted for publication) mimic. Even if the ECC genome were responsive to the molecules which Friend cells use to maintain their epigenetic state, it is not certain that these influences would survive the "unnatural" early stages of hybrid formation. Indeed, the experiments (reviewed in Gurdon, 1974) which have shown that pluripotent amphibian oocytes can "reset" the genomes
of differentiated nuclei inserted into oocyte cytoplasm hint that mammalian pluripotent cells, too, might perhaps be able to abrogate the epigenetic set of a mature differentiated cell.

As discussed above, several hybrid clones between teratoma-derived cells and fibroblasts have been reported; in general, these hybrids have resembled their fibroblast parents. PCT and PCF hybrids provide especially favorable material to test the generality of the notion that hybridization of ECCs destroys their pluripotency. For one thing, PCC4azal, FBU, and thymocytes all contain 39-40 chromosomes (and 40-43 chromosome arms); thus, considerations of gene dosage ought to play less of a role in these hybrids than in hybrids whose fibroblast parent contained 50-55 chromosomes (and roughly 55-65 chromosome arms). Perhaps more significantly, the genomes of PCC4azal and FBU (and, of course, normal thymocytes) closely resemble those of normal mouse cells. Aneuploid fibroblast cultures continually undergo karyotypic modification (Allerdice et al., 1973; Hashmi et al., 1974), so much so that of 11 A9 cells analyzed in the former paper, "no two cells had the same chromosome content." ECC cultures, in contrast, are remarkably stable (McBurney, 1976), and undergo little change in either chromosome number or morphology upon continued passage. Friend cells have not been so extensively analyzed, but their stable, near-diploid chromosome number and low content of bi-armed chromosomes suggest that they, too, resemble normal cells genetically. The rapid karyotypic evolution of most aneuploid cultures may well have facilitated selection for cells well adapted to growth in vitro, but may in the process have produced cells differing from normal ones in ways important to epigenetic mechanisms. It is important that developmental lessons
drawn from studies of hybrids derived from karyotypically unstable cells be tested independently.

Finally, embryonal carcinoma cells are interesting in their own right. They differ from most other cells not only in that they are pluripotent, but in their peculiar surface antigens, their resistance to many viruses, and in the ability of some ECCs to produce either tumors or normal mice depending on environment. ECC hybrid cells ought to give us a chance to test whether any of these properties can be dissociated from the others with which it is usually linked.
CHAPTER TWO: EXPERIMENTAL PROCEDURES

PART 2A: Materials

Unless otherwise stated, all biochemicals used were purchased from Sigma Biochemical Co. (St. Louis, Mo.); tissue culture media from Grand Island Biological Co. (Grand Island, N.Y.); serum for tissue culture from either Grand Island, Flow Laboratories (Rockville, Md.), or Microbiological Associates (Bethesda, Md.); radioactive chemicals from New England Nuclear (Boston, Ma.); materials for electron microscopy from Polysciences (Warrenton, Pa.); and plastic flasks and dishes for tissue culture from either Falcon (division of B.-D. Laboratories, Los Angeles, Ca.) or Corning (Corning, N.Y.).

PART 2B: Cell culture

Unless otherwise indicated, cells were grown in Dulbecco-Vogt modified Eagle's medium, "high glucose" formulation, which was purchased as a powder and made up in triply glass-distilled water, then filtered through Millipore (Bedford, Ma.) filters with final pore size .22 μm, and then supplemented with penicillin, streptomycin and kanamycin (final concentrations of 50 U/ml, 50 μg/ml, and 100 μg/ml, respectively) and 10% fetal calf serum. This medium will be referred to as DVME-FC, and serum-free medium as DVME-o. Hybrid cells were selected and grown in DVME-FC-HAT, produced by adding hypoxanthine, aminopterin (Nutritional Biochemicals, Cleveland, Ohio), and thymidine (final concentrations 13 mg/L, 0.19 mg/L, and 3.9 mg/L, respectively), prepared
as a 100x concentrate, to DVME-FC. Hepes buffer (20 mM, pH 7.6-7.8) was sometimes added to media in which the hybrid cells were grown.

Embryonal carcinoma cell lines, as well as PCT and PCF hybrid lines, were routinely fed on the second day after subculture, and split again at a ratio of 1:10 or 1:20 on the third day, using Viokase enzyme mixture (Grand Island) in a calcium- and magnesium-free balanced salt solution which contained 1 mM Na_4EDTA. Other cell lines required less frequent subculture. All cells were grown at 37° in a humidified atmosphere of 10% CO_2 in air.

PART 2C: Production of hybrid cells

PCT hybrids.

A suspension of thymocytes was prepared by teasing the cells from freshly-dissected thymus glands of two four-week old C3H/HeJ female mice into DVME-FC. These cells were washed and then resuspended in DVME-o at 2 x 10^7/ml and chilled to 4°. Logarithmically-growing PCC4azal cells were harvested with Viokase solution, washed once in DVME-FC and once in DVME-o, and resuspended at 10^7/ml. The fusion mixture contained 0.5 ml of each cell suspension and 1 ml of Dulbecco's phosphate-buffered saline containing 1% bovine serum albumin and 500 hemagglutinating units of β-propiolactone-inactivated Sendai virus prepared by the method of Giles and Ruddle (1973). This mixture was kept on ice for 20 min. and then swirled at 37° for another 65 min. The cells were then washed once in DVME-FC and seeded into Falcon 25 cm^2 flasks in DVME-FC-HAT.
PCC4azal cells are very sensitive to HAT, and did not survive the fourth day of incubation; nearly all thymocytes had died by then as well. By the ninth day of incubation, each of three flasks was found to contain a single healthy colony of attached cells. These flasks were fed on day 9, and the medium which was removed from the flasks at this (and several later) feedings cultured at 37° in the hope of recovering live non-attached cells. No growth was ever seen in the cultured fluids, but two of the three attached colonies gave rise to proliferating cell lines, which were named PCTA1 and PCTA2.

A second independent fusion was carried out in the same way, except that a) only $2.5 \times 10^6$ PCC4azal cells were used; b) the thymocyte donor was a 6-week old C3H/HeJ female; and c) the incubation at 37° was stopped after only 30 min. Two clones of attached cells were derived in this experiment: PCTBla and PCTB1b. Both lines were derived from the same flask, and may have evolved from the same fusion product. A third colony was lost because of poor growth, and no non-attached cells could be recovered from the culture fluids retained from the early feedings.

PCF hybrids.

Five clones of PCF hybrid cells were produced in two separate fusion experiments between PCC4azal and the BrdU-resistant, DMSO-inducible Friend erythroblastic leukemia line, FBU. In the first of these, $4 \times 10^7$ PCC4azal cells were mixed with $8 \times 10^6$ FBU cells. The FBU cells, since their original isolation, had been grown in medium containing 30 μg/ml BrdU to select against revertants, and had been
grown in medium containing 2% DMSO for three days prior to the fusion experiment. 1 ml of Sendai virus (500 HAU in Dulbecco's PBS with 1% bovine serum albumin) was added to the 1 ml of DVME-o in which the mixture of cells had been resuspended. This mixture was then kept at 4° for 10 min, and incubated at 37° for another 20 min. The cells were then seeded into ten 25 cm² flasks, and HAT additives were added on the following day. Of the several colonies of attached cells which appeared within the next two weeks only one, PCFA8, survived for further characterization.

A second experiment was carried out in a similar way, except that the FBU culture had not been treated with DMSO prior to fusion. Four attached colonies of cells, all derived from separate flasks and hence presumed to have originated in different fusion events, were isolated for characterization: PCFB1, PCFB2, PCFB3, and PCFB5.

Several flasks in these experiments were found to contain proliferating populations of non-attached cells. Clones obtained by seeding these cells at a concentration of 1 cell/well in Falcon microtest plates were found (by isozyme analysis) to be Friend cell revertants. It is possible that the non-attached populations originally contained hybrid cells which were overgrown by FBU revertants and therefore not recovered in the cloning experiments.

PART 2D: Starch gels and chromosome analysis

Starch gel electrophoretic analysis.

Sonicates prepared from cultured cells and from homogenized mouse

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tissues were analyzed for isozyme content essentially by the methods of Nichols and Ruddle (1973), except that harvested cells were sonicated during the course of sample preparation.

Chromosome analysis.

Rapidly dividing cultures of hybrid and parental cell lines were treated with Colemid (Ciba) at concentrations of 0.03-0.06 µg/ml for 1-4 hours, harvested, swollen in 0.075 M KCl or in fetal calf serum diluted with 3-5 volumes of distilled water, fixed in 3:1 methanol: acetic acid, and air-dried onto clean slides. These preparations were stained with Giemsa or the fluorochrome 33258 Hoechst, unbroken and well-spread metaphase preparations photographed, and chromosome counts made either from the prints or from projected negatives.

PCC4azal cells contain two bi-armed chromosomes, of which one (marker "P-1") is longer than any of the four bi-armed chromosomes found in FBU cells. FBU metaphases, on the other hand, contain two (probably homologous) submetacentrics (F-2) and two (probably non-homologous) metacentrics (F-1) which can be distinguished from the remaining PCC4azal metacentric (P-2) by staining with 33258 Hoechst. Most mouse centromeres fluoresce brightly after Hoechst staining, but the centromere of marker P-2 is much less bright than either of the Friend F-1 markers. No attempt was made to identify specific chromosomes by banding techniques.

PART 2E: Electron microscopy

Monolayers of hybrid cells growing logarithmically in plastic
tissue culture dishes were prepared for electron microscopy by the method of Brinkley and Chang (1973). Glutaraldehyde-fixed, osmium post-fixed monolayers were dehydrated and embedded in Epon, sectioned, stained with uranyl acetate and lead citrate, and then examined and photographed with a Phillips 300 microscope.

PART 2F: Measurement of alkaline phosphatase activity

Alkaline phosphatase activity (E.C. 3.1.3.1; APase) was measured in cell sonicates by spectrophotometric quantitation of the hydrolysis of p-nitrophenyl phosphate during a 15 min incubation at 37° and pH 10.0. The assay is essentially the same as that used by Bernstine et al. (1973), and the results are expressed in the same units employed by these workers. To confirm that my results could be compared directly with the values published by Bernstine's group, I measured APase in mouse kidney sonicates. As shown below (see Table 3-3), the average specific activity I obtained for this tissue (31.5 μmol/hr/mg protein; s.e.m. 3.4) is similar to that obtained by Bernstine (28.0; range 21.8-38.0). I assayed each sonicate in duplicate on the day of preparation, and the duplicate values were averaged to obtain a specific activity for that day's enzyme preparation. The means shown in Tables 3-3 and 3-4 are thus averages of different daily preparations.

PART 2G: Infection of cultured cells by Minute Virus of Mice

The Crawford strain (Crawford, 1966) of the parvovirus MVM
("Minute Virus of Mice") was obtained from Dr. D. Ward as a suspension at $5 \times 10^7$ plaque-forming units (PFU)/ml in DVME with 5% fetal calf serum, together with a fluorescein-conjugated rabbit antiserum to MVM virions. Details of virus preparation and titration, and antiserum production and conjugation are given in Miller, Ward, and Ruddle (1976; submitted for publication.)

Cells which were to be tested for the ability to support MVM infection were seeded in counted numbers onto Lab-Tek 8-chamber slides (Miles Laboratories) at $1-3 \times 10^4$ cells/cm$^2$ chamber. After an overnight incubation to permit the cells to attach, each monolayer was washed once in DVME-o. Virus was added in 0.2 ml of DVME-o, at a multiplicity of 20 PFU/plated cell. (Assuming one population doubling between subculture and virus addition, this gives an effective multiplicity of infection (MOI) of 10.) The virus was removed 1 hr later, the appropriate serum-containing medium added (0.3 ml/chamber), and incubation continued at 37° for 15-20 hr.

Virus-treated cell cultures were then tested for the presence of MVM antigens by an indirect immunofluorescence assay. Slides to be tested were washed briefly in PBS at room temperature, fixed in 3:1 methanol:acetone for 10 min at room temperature, and air-dried. A drop of anti-MVM serum, diluted 1:40 with PBS, was placed on the slide and covered with a coverslip to distribute the serum evenly over all eight chambers. After 30 min at 37°, the slide was washed in three changes of PBS, shaken to remove most of the PBS, and covered in the same way with a fluorescein-conjugated IgG fraction of goat antibody to rabbit IgG (Microbiological Associates), diluted 1:20 in PBS. After a second 30 min incubation, the slides were washed in three changes of PBS,
counterstained with 0.005% Evans Blue in PBS with 1% FCS for 30–60 sec, and rinsed in water. Fluorescent preparations were examined and photographed with a Leitz Orthoplan microscope using mercury illumination deflected through a Ploemopak vertical fluorescence illuminator carrying a filter system designed to provide wide-band blue illumination (Leitz Filter System H, which includes two KP490 excitation filters, a TK510 dichroic prism, and a K510 barrier filter).

Cells which grew in suspension (FBU and concanavalin-stimulated mouse splenic lymphoid cells) were infected at an MOI of 10 and a cell concentration of $2 \times 10^5$/ml in DVME-FC (FBU) or $10^6$/ml in RPMI-FC (lymphoid cells). After 18–19 hr, the cells were washed in DVME-o, resuspended, and a drop of suspension placed on a microscope slide and air-dried. These slides were then fixed and stained as described above.

PART 2H: Production of tumors from hybrid cells

Cells were harvested in 0.02% Na$_4$EDTA in PBS, washed in DVME-o, and injected in counted numbers subcutaneously into young adult mice. Some of the tumors were grown in homozygous nu/nu mice (partially inbred onto a Balb/c background; Freedman and Shin, 1974), which are genetically deficient in their cell-mediated immune response. Other tumors were grown in syngeneic animals: PCT cells in (C3H/HeJ x 129/Sv)$F_1$, and PCF in (DBA/2J x 129/Sv)$F_1$ mice. The nude mice were raised and inoculated by Dr. S. Shin at Albert Einstein College of Medicine, and the syngeneic animals bred and inoculated here at Yale. Both kinds of host animals produced similar sorts of tumors. After
17–45 days, animals with tumors were killed by cervical dislocation, and their tumors removed, fixed in aqueous Bouin's fluid, embedded in paraffin, sectioned at 4–6 µm, stained with hematoxylin and eosin, and examined with a Leitz Orthoplan microscope.

To learn whether tumor formation had been accompanied by changes in chromosome complement, I prepared cultures from 4 of the PCT tumors at autopsy, from which chromosome preparations were made 3–4 sub-culturings after derivation.

PART 2I: α-fetoprotein (AFP) production by hybrid tumors

Blood was drawn from some of the tumor-bearing animals just prior to sacrifice. Serum from these mice was tested, after storage at -50°, for the presence of mouse AFP by the discontinuous counterimmunoelectrophoretic technique of Wallis and Melnick (1971, 1972). Microscope slides were coated with 1% agarose (Seakem) gel containing 12 mM barbital buffer (pH 8.6), and wells punched into the agarose were loaded with about 5 µl of the antigen to be tested, diluted 1:4 with buffer. Rabbit antiserum to mouse AFP was pipetted into the cathodal series of wells. Slides were then subjected to electrophoresis at 200 volts/8 cm slide, for 30 min at room temperature. Reservoir and bridge buffers were at five-fold higher buffer concentration. The antiserum used (the gift of Dr. B. Ledford) was produced in a rabbit injected with electrophoretically purified AFP isolated from a cultured mouse hepatoma line, Hepa. The serum was absorbed with mouse albumin prior to use. I have tested the specificity of this serum in several ways. First, it gives a single precipitin line in a standard immunoelectro-
phoretic assay when tested against either mouse amniotic fluid, Hepa
culture fluid, or serum from a mouse carrying an OTT6050 ascitic 
teratocarcinoma; the antiserum, however, produced no precipitin line 
when tested against adult mouse serum. Second, when this antiserum is 
compared with a second, independent anti-AFP serum, produced by Dr. E. 
Zimmerman by immunization of a goat with mouse amniotic fluid-derived 
AFP, a line of identity is produced in double diffusion analysis, 
using mouse amniotic fluid as antigen. I used the rabbit antiserum 
to mouse AFP at a dilution of 1:4 in 12 mM barbital buffer.

After discontinuous counterimmunoelectrophoresis, the agarose-
covered slides were washed for 2-4 days in several changes of saline, 
washed once overnight in distilled water, dried, stained with 0.025% 
Coomassie Blue B in a mixture of methanol, water, and acetic acid 
(45:45:10), and destained in the same solvent mixture.

PART 2J: Production and titration of anti-Thy-1 alloantiserum

An alloantiserum to detect the presence of Thy-1.2 antigen was 
prepared by a method based on that of Reif and Allen (1964a). Strains 
AKR/J and C3H/HeJ are both H-2^k, but differ at the Thy-1 locus, so 
that immunization between these strains produces a serum uncontaminated 
by anti-H-2 antibody. Such sera may, however, contain antibodies 
directed against molecules unrelated to Thy-1 antigen.

AKR/J female mice (7 weeks old when first inoculated) were injected 
intraperitoneally, once a week, with a suspension of C3H/HeJ female 
thymocytes, at a concentration of 10^7 cells/0.2 ml inoculum. The 
recipient mice were bled from the tail 6 and 10 days after the fifth
Injection. Antiserum was heat-inactivated (to remove complement) at 54° for 30 min, and stored in aliquots below -50°.

This antiserum was titred in a one-step cytotoxic test against C3H/HeJ thymus target cells. Equal volumes of diluted antiserum, diluted rabbit serum (as complement source), and cells (2 x 10^6/ml) were mixed and incubated at 37° for 45 min, after which trypan blue was used to assess cell death. The serum, in the presence of complement, killed more than 95% of the target cells at high concentrations, and killed 53% at a dilution of 1:1280. DMEM-o with 4% heat-inactivated fetal calf serum was used as diluent.

When titred in the two-step cytotoxic test employed (see below) for absorption analyses on ECC and hybrid cells, this serum killed more than 50% of the target C3H thymocytes at a dilution of 1:2000.

PART 2K: Cytotoxic absorption tests for cell surface antigens

Hybrid cells and appropriate controls were tested for the presence of H-2 and Thy-1 cell surface antigens by a modification of a standard method (e.g. Artzt and Jacob, 1974) involving absorption of cytotoxic antibody from well-defined sera. In the usual test, the antiserum is first titred for cytotoxic activity against target lymphoid cells by mixing counted numbers of target cells with a series of dilutions of the antiserum. After a period of sensitization, during which antibody binds to the cells, complement (C') is added. After a further 45 min incubation at 37°, the fraction of the target cell population still viable is measured, e.g. by counting the proportion still able to exclude a dye, trypan blue, which only enters dead cells.
After this titration, cells of interest can be tested for antigen by an absorption assay, as follows. A fresh aliquot of the antiserum is diluted to the lowest concentration found to kill 60-90% of the target cells. The diluted serum is then mixed with the cells in whose surface antigens one is interested. After an incubation, the cells are spun out, and the absorbed serum tested for residual cytotoxicity against target cells. What one learns from the absorption test is whether the tested cells carry antigens which cross-react with those both a) present on the target cell, and b) recognized by the antiserum used.

In a series of preliminary experiments not to be discussed here in detail, I observed that when this standard cytotoxic test was used, embryonal carcinoma cells and their hybrids often seemed to absorb cytotoxic antibody even from sera from which no absorption ought to have been observed. F9 cells, for example, absorbed cytotoxic anti-H-2\(^k\) antibody, even though they did not carry the specificities detected by the serum used. Several attempts to circumvent this non-specific absorption—e.g., by pretreating the F9 cells with bovine serum albumin, or pre-absorbing the anti-H-2 serum with F9 before use—all failed to prevent absorption, by F9 cells, of cytotoxic activity in excess of the small amount absorbed by negative control cells. In addition, I found that F9-absorbed sera were often more cytotoxic if diluted 1:2 than if used without dilution.

This last observation suggested that absorption of sera with ECCs might be rendering the sera anti-complementary. Were this the case, one would expect that F9-absorbed sera would always be less cytotoxic than control-absorbed aliquots, not because the F9 cells had removed...
antibody, but rather because F9-absorbed serum, when mixed with C', in some way inhibited the C'-dependent cytotoxic reactions. The increase in cytotoxic activity after a 1:2 dilution could be due to such an inhibitory influence if it acted with different stoichiometry than did antibody. Interference from anti-complementary activity could be overcome by modifying the cytotoxic test to prevent contact between C' and the absorbed serum. When such a modified test was carried out (see below), I began to obtain consistent results with acceptably low absorption in cases where ECCs were used as negative controls.

The cytotoxic test used to produce the results reported below was therefore carried out as follows:

1. Cells to be tested for surface antigen content were freed from monolayer cultures by two washes in PBS, followed by a 5 min incubation in PBS with 0.02% Na$_4$EDTA. Cells were shaken free, poured into an equal volume of cold diluent (DVM with 4% heat-inactivated fetal calf serum), sedimented at 4° (800 rpm, 4 min in an IEC model PR-6 with a #253 head), and resuspended in diluent. The cells were then packed by centrifugation at 1500 rpm for 5 min.

Lymphoid cells to be tested were teased from the appropriate organs into diluent, washed twice, and pelleted at 1500 rpm for 5 min.

Cultured suspension cells were prepared in the same way as lymphoid cells, except that they were washed three times. In an experiment not shown, PBS-EDTA treatment of suspension cells was shown not to diminish detectably their ability to absorb anti-H-2 antibody.

2. Absorption. An appropriate alloantiserum (see below) was diluted to a level just sufficient to kill 60-90% of target cells, and stored frozen in aliquots containing enough serum for one day's use.
50 μl of diluted serum were pipetted into each of a set of 400 μl tubes. The pellet of cells prepared in step #1 above was carefully freed of surface diluent, and 25 μl of packed cells were transferred to the tubes of serum. The cells were dispersed in the serum by gentle agitation, and the tubes incubated at 4°C for 40 min, after which the cells were pelleted in a Beckman microfuge.

3. Target cells were prepared by teasing the mesenteric lymph node (for H-2 assays) or thymus gland (for Thy-1 assays) into diluent. Cells were triturated, washed three times in the cold, counted, and suspended at 2 x 10^6/ml.

4. 20 μl each of diluent, absorbed antiserum, and target cell suspension were pipetted into 3 ml conical tubes kept at 4°C. Each absorbed serum sample was divided into two 20 μl portions and thus assayed in duplicate. Each series of tubes included two in which diluent was substituted for antiserum ("C' only control") and two in which unabsorbed serum was used. Each batch of diluted antiserum was tested to verify that cell death in its presence was in fact C'-dependent, but this control was not repeated for each day's work. These tubes were incubated for 40 min in an ice-water bath.

5. After this sensitization, target cells were washed in 2.5 ml of diluent and sedimented (10 min, 800 rpm, 4°C). Supernatant was removed except for an estimated 25 μl covering the pellet. 50 μl of rabbit C', absorbed with A9 cells as described below, and diluted to a concentration found to be optimal for each batch (ranging from 1:8 to 1:12), was then added. The cells were resuspended, warmed quickly to 37°C, and incubated for 45 min at this temperature.
6. The tubes were then placed in an ice-water bath, and 50 µl of .20% trypan blue in saline was added before an aliquot was examined in a hemocytometer. (The trypan blue was stored as a .25% solution in water, and mixed with .25 vols of 4.2% NaCl just before use.) 100 cells were counted from each tube.

Calculations.

The fraction of cells killed by a particular aliquot of absorbed serum is not itself the most useful measure of the ability of the absorbing cells to remove antibody, since this value is subject to several influences which suffer day-to-day variations: e.g. the initial viability of the target cell population, susceptibility of a given mouse's cells to C', minor diluting errors, incubator temperature, etc. For this reason, the counted fraction of viable cells was converted into a measure of "residual cytotoxicity" as follows:

1. Cytotoxic index (CI) was calculated as

\[ \frac{T - C}{100 - C} \]

where \( T \) is the fraction killed in the serum being tested and \( C \) is the fraction killed in the C'-only control.

This figure expresses killing as a fraction of the live cells not killed by C' alone, and compensates for variations in C' activity, initial cell viability, and susceptibility to C'. The CI for the C'-only control is by definition zero.

2. Residual cytotoxicity (RC) is calculated for each absorbed
serum as

\[
\frac{100 \times CI (T)}{CI (U)}
\]

where CI (T) is the cytotoxic index for the absorbed serum being tested and CI (U) is the cytotoxic index for the unabsorbed control. The RC for the unabsorbed control is thus by definition 100.

Complement.

Complement (C') for use in cytotoxic tests was prepared by the method of Boyse et al. (1970). Rabbit serum (either drawn from selected rabbits by cardiac puncture or purchased frozen from Grand Island Biological Co.) was made 10 mM in Na₂EDTA by addition from a 10x stock. The serum was then absorbed with A9 cells (washed several times in DMEM-o) at 4°C for 30 min with occasional agitation. Different batches of C' were absorbed with packed volumes of A9 cells ranging from 10-25% of the C' volume. During the absorption, naturally-occurring rabbit antibody to mouse cell surface antigens is removed, while the EDTA prevents consumption of C'. After the absorption, the C' was separated by centrifugation at 4°C for 5 min at 2000 g, and enough 20 mM CaCl₂ added to compensate for the EDTA. Absorbed C' was stored in 0.5 ml aliquots at -90°C; once thawed, an aliquot was never refrozen for later use.

Specificity.

It is important to recognize the limitations of specificity in this
technique as used with currently available antisera, and the tricks which are used to improve specificity. If, for example, one produces an "anti-H-2\(^k\)" serum by injecting A9 cells (C3H/HeJ; H-2\(^k\)) into Balb/c mice (H-2\(^d\)), and then uses this serum to kill A9 cells in a cytotoxic test, one will be able to detect H-2\(^k\) antigens on the surfaces of cells used for absorption. Such a system is, however, not specific for H-2 antigens, since the serum may well contain antibodies to other surface molecules (viral antigens, minor H antigens, etc.) present both on the immunizing and target cells. The principal precaution used to increase the specificity of cytotoxic tests is to use co-isogenic mice throughout. To test for H-2\(^k\), for example, one might produce an antiserum in C57BL/10 ("B10") mice by injection of lymphoid cells from the co-isogenic strain B10.BR, which differs genetically from its parental strain B10 only by the introduction of genes closely linked to the H-2\(^k\) complex introduced from the C57BR strain. Such an antiserum, if tested on B10.BR target cells, will only detect surface antigens coded for, or controlled by, genes closely linked to the major histocompatibility complex (MHC) and the H-2 loci.

It is worth noting that such a test system is still not entirely specific for any single antigen. Among the complicating factors are the following:

1. H-2 haplotypes contain two loci, called K and D, which produce highly immunogenic cell surface antigens. An antiserum can often be made specific for one or the other of these loci by the use of appropriate strains, one of which has undergone intra-MHC recombination. Both of the H-2 antisera used in this study (see Table 2-1) are of this type, and are directed against H-2K molecules.
<table>
<thead>
<tr>
<th>Antigen</th>
<th>Serum</th>
<th>Immunogen</th>
<th>Responder</th>
<th>Target</th>
<th>H-2 Specificities Detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>D33</td>
<td>B10.A (5R)</td>
<td>(B10.D2 x A)&lt;sub&gt;k&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;&lt;sub&gt;D&lt;/sub&gt;&lt;sup&gt;d&lt;/sup&gt;</td>
<td>C57BL/10</td>
<td>33, 53, 54 (H-2&lt;sup&gt;K&lt;sub&gt;b&lt;/sub&gt;&lt;/sup&gt;)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>K&lt;sup&gt;d&lt;/sup&gt;&lt;sub&gt;D&lt;/sub&gt;&lt;sup&gt;d&lt;/sup&gt; x K&lt;sup&gt;k&lt;/sup&gt;&lt;sub&gt;D&lt;/sub&gt;&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H-2&lt;sup&gt;k&lt;/sup&gt;</td>
<td>D3b</td>
<td>C3H&lt;sub&gt;k&lt;/sub&gt;&lt;sup&gt;k&lt;/sup&gt;</td>
<td>(C3H-H-2&lt;sup&gt;0&lt;/sup&gt; x 129)&lt;sub&gt;k&lt;/sub&gt;&lt;sup&gt;D&lt;/sup&gt;&lt;sub&gt;k&lt;/sub&gt;</td>
<td>B10.BR</td>
<td>11, 23, 25, 52 (H-2&lt;sup&gt;K&lt;sub&gt;k&lt;/sub&gt;&lt;/sup&gt;)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>K&lt;sup&gt;d&lt;/sup&gt;&lt;sub&gt;D&lt;/sub&gt;&lt;sup&gt;k&lt;/sup&gt; x K&lt;sup&gt;b&lt;/sup&gt;&lt;sub&gt;D&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;&lt;sub&gt;c&lt;/sub&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>K&lt;sup&gt;D&lt;/sup&gt;&lt;sub&gt;k&lt;/sub&gt;&lt;sup&gt;k&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Details of the immunization procedures are given in Snell (1968). These sera were obtained through Dr. John Ray of the Collaborative Research office of the National Institute of Allergy and Infectious Disease, NIH. The underlining indicates the molecule that the antiserum was designed to detect; as noted in the text, these antisera may contain antibodies directed against other molecules as well.
2. Cross-reactions, due to the so-called "public specificities", may occur between different individual H-2 antigens. Reference to a list of H-2 specificities (Klein, 1975, p. 126) can help one to decide whether a particular antiserum-target cell combination can detect a public specificity likely to be present on the cell whose surface composition one wishes to examine.

3. Recent work has shown that the MHC includes genes which code for surface molecules other than H-2K and H-2D; these new antigens are called Ia antigens (for review, see Klein, 1975, pp. 450-54. For recent information on strain distributions, see Götze, 1976.) In addition, it is likely that the MHC contains loci coding for surface antigens still to be recognized. Anti-H-2 sera, even those produced through immunization of co-isogenic strains, may be "contaminated" with antibodies to the products of these loci. It is also possible that some of the MHC gene products act to control the expression of surface antigens coded for in other areas of the genome.

Table 2-1 summarizes the cytotoxic systems used in this dissertation to test for H-2K\(^b\) and H-2K\(^k\) antigens. For the reasons just stated, a cautious interpretation of results obtained using such sera would be that although most of the absorption of cytotoxic activity brought about by a test cell is likely to represent classical H-2K determinants on the cell surface, one cannot rule out absorption due to the products of loci which are linked to H-2 but are not yet well-defined.

PART 2L: Measurement of acetylcholinesterase activity

Acetylcholinesterase (E.C. 3.1.1.7; AChE), an enzyme present at
high specific activity in erythrocytes, is also found in Friend erythro-
leukemia cells (Conscience et al., submitted for publication), but not
in embryonal carcinoma cells (see below). PCF hybrid clones were
tested for AChE activity essentially by the method of Lewis and
Eldefrawi (1974). Harvested cells were sonicated in 50 mM phosphate
buffer, (pH 7.4, 1% in NP40 detergent); the sonicate was clarified by
centrifugation (30,000 g, 30 min, 4°) and divided into aliquots which
were frozen until use. The reaction was carried out in a total volume
of 150 μl, and is based on the measurement of radioactive acetate re-
leased from acetylcholine substrate during a 20 min incubation at room
temperature. Acetate is separated from unhydrolyzed substrate by thin
layer chromatography on strips cut from Gelman Type SA ITLC plates,
using 80% ethanol as solvent, which are tested for radioactivity. Each
set of assays included duplicate determinations for each aliquot, as
well as blanks in which 1% NP40 in buffer was substituted for the
enzyme solution. The percent hydrolysis given by the blank was sub-
tracted from each result before further calculation; this correction
averaged 6-8% hydrolysis, and was in most cases roughly 10-fold greater
than the hydrolysis attributable to enzyme activity in ECC and PCF
sonicates (blank-corrected), though of course much lower than the
hydrolysis produced by FBU sonicates.

PART 2M: Other methods

Friend cells and cells harvested from monolayer culture were
stained for hemoglobin by the benzidine method of Orkin et al. (1975).
Two drops of benzidine solution were added to a single drop of cell
suspension in a well of a Falcon microtest plate.

Protein was measured by the method of Lowry et al. (1951), using bovine serum albumin as a standard, during the course of enzyme assays.
PART 3A: Demonstration that PCT and PCF cells are in fact hybrids

PCT cells.

As described in Part 2C, above, two monolayer cell lines, PCTA1 and PCTA2, were derived from a Sendai-mediated fusion between PCC4azal and C3H/HeJ mouse thymus cells. A second, similar fusion produced two other cell lines, PCTBla and PCTB1b, which appeared as separate colonies in the same flask, and might therefore have been derived from a single fusion product. Attempts to isolate lines of non-attached cells from the fusion mixtures were unsuccessful.

Because these cell lines grew in a medium (DVME-FC-HAT) in which neither parent was able to divide, they were presumed to be hybrids. Two experiments were carried out to test this presumption.

Sonicates of each of the four cell lines, together with appropriate controls, were checked by starch gel electrophoresis for their pattern of glucose phosphate isomerase (GPI; E.C. 3.5.1.9) isozymes. Figure 3-1 shows that each of the four cell lines contains both the GPI-A marker characteristic of the PCC4azal parent and the GPI-B enzyme found in the C3H/HeJ thymocyte parent, as well as an AB heterodimer found only in cells carrying alleles for both forms. A cell containing equal numbers of active A and B alleles would be expected to produce the three isozymes in ratios of 1:2:1 (AA:AB:BB); the relative densities of the three bands in the PCT cell lines, as in the (C3H/HeJ x 129/J)F1 hybrid mouse thymus control, conform moderately well to this expectation. This
Figure 3-1

Glucose phosphate isomerase isozymes in PCT hybrids and controls.
Glucose Phosphate Isomerase

(-)  

(+)  

C3H thymus (C3H x 129)F1 PCC4aza1 PCTA2 PCTB1a PCTB1b PCTA1

B AB A

---Origin

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result shows that each PCT line contains cells with some genetic input from each parental cell, and suggests that each hybrid line arose from the fusion of one thymocyte with one ECC.

Chromosome analysis supported this idea. Figure 3-2a shows a metaphase preparation of a typical PCT hybrid cell, and figure 3-3 presents histograms of chromosome number for each of the PCT lines. Table 3-1 shows the summary statistics derived from the chromosome counts of these lines and of the PCC4azal parent. The expected values shown in this table are based on the assumptions that the thymus parent contained 40 acrocentric chromosomes, that the PCC4azal parent contained the modal number of its population (39) including both of the bi-armed marker chromosomes, and that no chromosome loss occurred after fusion from a 1:1 hybrid. The PCT hybrid populations contained 92-95% (means) of the expected chromosome number, and 80-90% of the hybrid metaphases showed both of the PCC4azal marker chromosomes. Although it is conceivable that extensive chromosome loss has taken place, accompanied by reduplication of the remaining chromosomes, it seems far more likely that each hybrid cell contains most of the chromosomes introduced by each parent.

The chromosome analysis, together with the GPI isozyme pattern, shows that these cell lines are products of fusions between single thymus cells and single PCC4azal cells. Each parent probably contributed about the same amount of genetic material to the initial hybrid cell.

The results of serological analyses to be presented below show that the PCT lines also contain a second marker from the thymus parent, H-2^k, and that at least three of the lines carry the H-2^bc allele found in the 129/Sv parent from which PCC4azal was derived.
Metaphase preparations of hybrid cells and parents.

a) PCTA1. Giemsa-stained. 71 acrocentrics; 1 P-1; 1 P-2.
b) PCC4azal. Hoechst-stained. 37 acrocentrics; 1 P-1; 1 P-2.
c) FBU. Hoechst-stained. 34 acrocentrics; 2 F-1; 2 F-2.
d) PCFB2 (passage 8). Hoechst-stained. 96 acrocentrics; 1 P-1; 2 P-2; 2 F-1; 2 F-2.

Code:  P-1: single triangle.
       P-2: double triangles.
       F-1: single arrow.
       F-2: double arrows.

Note that photographs shown are not to the same scale. Note the presence of two doses of PCC4azal marker P-2 in the PCFB2 metaphase shown.
Figure 3-3

Distribution of chromosome numbers in PCT hybrid populations.
### TABLE 3-1

**CHROMOSOME CONSTITUTION OF PCT HYBRID CELLS**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Mean #</th>
<th>S.D.</th>
<th>N</th>
<th>% With 2 Bi-armed</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCTA1</td>
<td>75</td>
<td>2.8</td>
<td>39</td>
<td>97</td>
</tr>
<tr>
<td>PCTA2</td>
<td>73</td>
<td>3.7</td>
<td>20</td>
<td>85</td>
</tr>
<tr>
<td>PCTBla</td>
<td>75</td>
<td>3.2</td>
<td>21</td>
<td>86</td>
</tr>
<tr>
<td>PCTBlb</td>
<td>74</td>
<td>3.3</td>
<td>23</td>
<td>87</td>
</tr>
<tr>
<td>PCC4azal</td>
<td>38</td>
<td>1.7</td>
<td>32</td>
<td>100</td>
</tr>
<tr>
<td>Thymocytes</td>
<td>(40)</td>
<td>---</td>
<td>--</td>
<td>(0)</td>
</tr>
<tr>
<td>Expected</td>
<td>79</td>
<td>---</td>
<td>--</td>
<td>100</td>
</tr>
</tbody>
</table>

Values given are the mean numbers of chromosomes found in N metaphases examined for each cell line, except that the values given for thymocytes were assumed. S.D. gives the standard deviation of the distribution. Expected values (for a 1:1 hybrid) are based on the assumptions given in the text. Values for mean and S.D. for PCC4azal were computed after excluding near-tetraploid cells which are, however, included in the histogram (figure 3-6). Note that the strong modal value for the PCC4azal population, 39, is slightly above the mean chromosome number.
PCF cells.

As described in Part 2C above, five monolayer cell lines were derived from two fusion experiments between PCC4azal and the Friend cell line FBU. A single clone, PCFA8, survived the first experiment; the second produced four independent clones: PCFB1, PCFB2, PCFB3, and PCFB5. Attempts to isolate lines of non-attached hybrid cells were not successful. The PCF cells grew well in DME-FC-HAT, and were tested further to see if they were truly hybrid.

The mouse strains, 129/Sv and DBA/2J, from which PCC4azal and FBU were, respectively, derived differ genetically with respect to the isozymes phosphoglucomutase (PGM: E.C. 2.7.5.1) and isocitrate dehydrogenase (IDH; E.C. 1.1.1.42). Each putative PCF hybrid clone was tested for PGM and IDH isozymes within 6-10 passages of its original isolation. Figure 3-4 shows that each of the PCF clones (together with a clone, PCFB4, which was subsequently lost) has both the two bands of PGM-1A activity characteristic of 129/Sv (PCC4azal) cells, and the two bands of PGM-1B activity of the DBA/2J (FBU) parent. Figure 3-5 shows similar results for IDH. PCC4azal has a slower-migrating isozyme, IDH-1A, while the faster IDH-1B form is characteristic of FBU cells. Each hybrid clone has both these isozymes, together with an AB heterodimer. It is worth noting that the A and B forms seem to be of equal intensity in PCFB3 and PCFB5 sonicates, but that sonicates of PCFA8, PCFB1, and PCFB2 have more of the PCC4azal form, and more dimer, than they have B isozyme. This suggestion of a greater ECC contribution to the latter three hybrid clones is confirmed by the chromosome data to be discussed next.
Figure 3-4

Phosphoglucomutase isozymes in PCF hybrids and controls.
Figure 3-5

Isocitrate dehydrogenase isozymes in PCF hybrids and controls.
Isocitrate Dehydrogenase

+ IDH -1 B
- IDH -1 A

Origin
IDH (mitochondrial)

FBU PCC49201 PCFA8 PCFB4 PCFB3 PCFB2 PCFB1
Figure 3-6 shows the distributions of chromosome numbers in metaphase spreads prepared from these cell lines. The results shown by open bars (for PCFA8 and PCFB2) were obtained 21-26 passages after the data indicated by the closed bars. Table 3-2 gives summary statistics condensed from the histograms and data on the presence, in the hybrid cells, of marker chromosomes characteristic of the two parental populations. The "expected" values are based on the assumptions mentioned above in connection with the PCT chromosome data, except that expected values for 2:1 (PCC4azal:FBU) hybrids are also given. Figure 3-2 (b,c,d) presents a Hoechst-stained metaphase spread of a typical PCF hybrid cell, together with similar preparations from each parental line.

The IDH data illustrated in figure 3-5 and discussed above suggests that PCFB3 and PCFB5 were each the product of fusion between equal numbers (presumably one) of PCC4azal and FBU cells. The chromosome numbers are consistent with the idea that these clones originated in 1:1 fusions, followed by loss of about 10% of the initial chromosome complement. On the basis of length, centromere position, and intensity of centromere staining with the fluorochrome Hoechst 33258, it is possible to distinguish from one another four kinds of bi-armed chromosomes in PCC4azal and FBU cells. Two of these (P-1 and P-2) are present in PCC4azal, and most of the PCFB3 and PCFB5 metaphases had a single copy of each of these markers. F-1 and F-2, similarly, are FBU markers, each represented in two examples in FBU cells. (It is not clear whether these represent two sets of similar but distinct chromosomes.) Most PCFB5 metaphases had two examples of each Friend cell marker, and most PCFB3 cells had two examples of F-2 and one of F-1.
Figure 3-6

Distribution of chromosome numbers and markers in PCF hybrids and parental populations.

Note that two separate chromosome preparations were performed on clones PCFA8 and PCFB2. The distributions indicated by the open bars were obtained 21-26 subculturings after those indicated by the closed bars.
<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Mean #</th>
<th>S.D.</th>
<th>N</th>
<th>Markers (&gt;30% of Cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCC4azal</td>
<td>38</td>
<td>1.7</td>
<td>32</td>
<td>1 1 0 0</td>
</tr>
<tr>
<td>FBU</td>
<td>39</td>
<td>1.9</td>
<td>29</td>
<td>0 0 2 2</td>
</tr>
<tr>
<td>Expected (1:1)</td>
<td>78</td>
<td>---</td>
<td>---</td>
<td>1 1 2 2</td>
</tr>
<tr>
<td>PCFB3</td>
<td>70</td>
<td>5.8</td>
<td>38</td>
<td>1 1 1 2</td>
</tr>
<tr>
<td>PCFB5</td>
<td>73</td>
<td>4.0</td>
<td>33</td>
<td>1 1,0 2 2</td>
</tr>
<tr>
<td>Expected (2:1)</td>
<td>117</td>
<td>---</td>
<td>---</td>
<td>2 2 2 2</td>
</tr>
<tr>
<td>PCFA8</td>
<td>94</td>
<td>9.7</td>
<td>16</td>
<td>1,2 2 1,2 1,2</td>
</tr>
<tr>
<td>PCFB1</td>
<td>107</td>
<td>10.3</td>
<td>34</td>
<td>2 1 2,1 2,1</td>
</tr>
<tr>
<td>PCFB2</td>
<td>97</td>
<td>12.1</td>
<td>18</td>
<td>2,1 2 2,1 2</td>
</tr>
</tbody>
</table>

For each cell line, N cells were counted to give a mean total chromosome number/cell, and a standard deviation (S.D.) was computed from the distribution. Four "marker" chromosomes could be distinguished on the bases of length, centromere position, and affinity for Hoechst 33258 dye. The numbers given for marker chromosomes are modal, and include all modes in which at least 30% of the cells of a given population joined. When two modes are given, the one listed first was the more common.

Assumptions used to compute expected values are as given in the text. Note particularly that the strong mode of the PCC4azal population, 39, is slightly higher than the mean number of PCC4azal chromosomes.
The data are consistent with the idea that these hybrids resulted from a 1:1 fusion.

Similar considerations support the hypothesis that PCFA8, PCFB1, and PCFB2 are the products of fusions between one Friend cell and two PCC4azal cells, although chromosome loss seems to have been more extensive than in the 1:1 hybrids. Note particularly the frequent presence, in metaphases of these three lines, of two copies of the PCC4azal bi-armed markers. The histograms for lines PCFA8 and PCFB2 shown in figure 3-6 support the notion that these cells can lose chromosomes during their period in culture.

In summary, and given the caveats discussed above with reference to the PCT lines, the chromosome and isozyme analysis of the PCF clones demonstrates that the cells are indeed hybrid, that PCFB3 and PCFB5 retain most of the chromosomes contributed by each genome, and that the other three PCF clones, after formation from the fusion of two PCC4azal cells with a single FBU cell, progressively lose up to 25% or more of their original chromosome complement.

PART 3B: Morphology of PCT and PCF cells in culture

PCT cells.

Figure 3-7a shows what PCT cells look like when grown logarithmically as monolayer cultures on plastic substrates. Figures 3-7b and 3-7c show, for comparison, the two parental cell types. PCT cells, like their ECC parent, grow as clusters of loosely attached cells. The ratio of nuclear volume to cytoplasmic volume is high compared to that of many
Figure 3-7

Morphology of cultured PCT and parental cells.

a) PCTBla. Phase contrast; 330x.
b) PCC4azal. Phase contrast; 330x.
c) Mouse thymocytes. Phase contrast; 330x.
d) PCTA1. Transmission electron micrograph; 4900x.
other cultured cell types. By phase contrast, the nucleus typically contains a single prominent phase-dense nucleolus within an otherwise phase-lucent nucleoplasm; there is very little chromatin emargination. Nuclei with two prominent nucleoli are more common in PCT cultures than in the PCC4azal parental line, probably because the former cells are near-tetraploid. Although no one of these traits is diagnostic of ECCs, together they make up a gestalt which is sufficiently compelling to have induced experts in teratoma cultivation (F. Jacob, personal communication) into mistaking cultured PCT cells for their PCC4azal parent.

The electron micrograph shown in figure 3-7d demonstrates that PCT cells closely resemble PCC4azal cells at ultrastructural resolution as well. The numerous free ribosomes, paucity of endoplasmic reticulum, lack of distinctive organellar apparatus, and predominance of euchromatin in the nucleus have all been described by other workers (see Damjanov and Solter, 1975) as characteristic of ECCs. Stobo et al. (1972) present several electron micrographs of murine thymocytes. Like ECCs, thymocytes have a high nucleus/cytoplasm ratio, few cytoplasmic organelles, and numerous free ribosomes. Thymocyte nuclei, however, unlike the nuclei of PCC4azal and PCT cells, contain large amounts of condensed chromatin, distributed throughout the nucleus but especially plentiful at the nuclear periphery. Paulin et al. (1976) have found biochemical correlates of the unusual nuclear morphology of ECCs; it would be interesting to test the composition of the nuclear proteins of PCT hybrid cells with this in mind. Thymus cells, of course, also differ from PCT and PCC4azal cells in that they do not form clumps and do not attach to the surface of tissue culture flasks.
PCF cells.

Figure 3-8 shows what cultured PCF cells look like, and shows, for comparison, phase contrast micrographs of the two parental cell types. Figure 3-8d is an electron micrograph of a section through a PCFB3 culture. Although PCF cells usually have more than one nucleolus (perhaps because they have more than one diploid equivalent of genome), the morphological similarities to PCT hybrid cells and PCC4azal cells are apparent. Some of the PCF cells in the cluster shown in figure 3-8a have formed a mound rising above the plastic surface into the medium; this growth habit is typical of many ECC cell lines, though not of PCC4azal itself. The electron micrograph shows that the PCF cells resemble ECCs at ultrastructural resolution as well. (The section shown happens not to have passed through a nucleolus.)

Sato et al. (1971) have discussed the ultrastructure of Friend cells as part of their study of DMSO-induced changes in these lines. They found that Friend cells, prior to any exposure to DMSO, and like PCC4azal and PCF cells, have high ratios of nuclear volume to cytoplasmic volume, and numerous free ribosomes. Friend cell cytoplasm also contains occasional lipid vacuoles and intracisternal A-type particles. The most obvious ultrastructural difference between Friend cells, on the one hand, and ECC and PCF cells on the other, is in nuclear morphology. Friend cells have large amounts of emarginated heterochromatin, and two nucleoli which, in stained preparations, are much less prominent than the single ECC nucleolus. Cultured Friend cells also neither clump nor attach to plastic surfaces (although one can select for weakly-attached Friend cell populations.)
Figure 3-8

Morphology of cultured PCF and parental cells.

a) PCFB2. Phase contrast; 330x.

b) PCC4aza1. Phase contrast; 330x.

c) FBU. Phase contrast; 330x.

d) PCFB3. Transmission electron micrograph; 6000x.
In view of the fact that other teratocarcinoma hybrids, including hybrids with neuroblastoma cells (Evans, 1975), are said to have resembled fibroblasts, it is worth noting that PCT and PCF hybrids clearly do not resemble cultured fibroblasts morphologically.

The tissue type which predominates in poorly differentiated areas of PCT- and PCF-derived tumors also closely resembles embryonal carcinoma tissue (see Part 3E, below.)

In summary, then, PCT and PCF cells resemble their ECC parent ultrastructurally, in their phase contrast appearance, and in their growth habit.

PART 3C: Alkaline phosphatase levels in PCT and PCF cells

Table 3-3 shows the results of a series of APase determinations carried out on third-day (near-confluent) cultures of PCT hybrid cells, together with measurements made at the same time on control materials. As explained above, these experiments were prompted by the demonstration of Bernstine et al. (1973) that cultured ECCs, like their counterparts in teratocarcinoma tumors, had high levels of this enzyme. To see if my results could profitably be compared with the values reported by this group, I included mouse kidney homogenates in many of the assays, and found that the value I obtained (31.5 μmol/hr/mg protein; s.e.m. 3.44) was similar to that obtained by Bernstine’s group (28.0; range 21.8-38.0).

The specific activities of APase in two ECC lines, F9 and PCC4azal, are presented in Table 3-3 as well. These levels are near the lower end of the range reported to be characteristic of ECC lines, but 10-20 times
### TABLE 3-3

ALKALINE PHOSPHATASE IN PCT HYBRIDS AND CONTROLS

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Specific Activity</th>
<th>S.E.M.</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCTA1</td>
<td>3.91</td>
<td>0.51</td>
<td>8</td>
</tr>
<tr>
<td>PCTA2</td>
<td>3.61</td>
<td>0.50</td>
<td>8</td>
</tr>
<tr>
<td>PCTBla</td>
<td>2.09</td>
<td>0.38</td>
<td>8</td>
</tr>
<tr>
<td>PCTBlb</td>
<td>2.50</td>
<td>0.38</td>
<td>8</td>
</tr>
<tr>
<td>PCC4azal</td>
<td>4.04</td>
<td>0.56</td>
<td>6</td>
</tr>
<tr>
<td>F9</td>
<td>5.09</td>
<td>0.53</td>
<td>7</td>
</tr>
<tr>
<td>TSD4</td>
<td>0.21</td>
<td>0.04</td>
<td>4</td>
</tr>
<tr>
<td>Thymocytes</td>
<td>0.53</td>
<td>0.18</td>
<td>7</td>
</tr>
<tr>
<td>Kidney</td>
<td>31.48</td>
<td>3.44</td>
<td>9</td>
</tr>
</tbody>
</table>

Specific activities are given in μMoles p-nitrophenyl phosphate hydrolyzed/hr at 37°/mg protein. The values given are the means and standard errors of the means of N separate daily preparations.
higher than the activities found in most other cultured cell lines (Bernstine et al., 1973). Data for one non-pluripotent line, TSD4, a fibroblast cell line derived from OTT6050 embryoid bodies, is presented in Table 3-3. Mouse thymus cells also contain less APase than ECCs. All four PCT hybrid lines have significantly more APase than either their thymocyte parents or TSD4; one of the four has significantly less enzyme than the PCC4azal parents, and the other three have slightly but not significantly less than their ECC parent (statistical analysis not shown). Histochemical experiments showed that nearly all cells in logarithmic PCC4azal and PCT cultures had detectable amounts of enzyme, and that the activity was concentrated, as is the case for embryonal carcinoma cells in tumors (Damjanov and Solter, 1975), at the intercellular boundaries.

Table 3-4 shows comparable data for the five PCF hybrid clones. These results were obtained several months after the ones shown in Table 3-3, and it is worth noting that the specific activity of the PCC4azal subline used as a control for the PCF experiments had somewhat more APase activity than the closely related line I had used earlier. The divergence in related sublines suggests that small differences in APase activity between ECC lines, even if statistically significant, may not be biologically important. All five PCF clones have as much or slightly more APase activity than their PCC4azal parent, even though FBU has no detectable APase.

In summary: PCT and PCF hybrid cells have high levels of alkaline phosphatase, an enzyme characteristic of embryonal carcinoma cells. The levels in the hybrids are comparable to those of PCC4azal, but much higher than those of the non-pluripotent parent in each case, and higher
### TABLE 3-4

**ALKALINE PHOSPHATASE IN PCF HYBRIDS AND CONTROLS**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Specific Activity</th>
<th>S.E.M.</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCFA8</td>
<td>9.94</td>
<td>1.37</td>
<td>5</td>
</tr>
<tr>
<td>PCFB1</td>
<td>7.22</td>
<td>1.52</td>
<td>5</td>
</tr>
<tr>
<td>PCFB2</td>
<td>9.54</td>
<td>0.78</td>
<td>5</td>
</tr>
<tr>
<td>PCFB3</td>
<td>10.15</td>
<td>1.41</td>
<td>5</td>
</tr>
<tr>
<td>PCFB5</td>
<td>10.88</td>
<td>0.88</td>
<td>5</td>
</tr>
<tr>
<td>PCC4azal</td>
<td>7.32</td>
<td>1.58</td>
<td>5</td>
</tr>
<tr>
<td>FBU</td>
<td>&lt;0.1</td>
<td>----</td>
<td>3</td>
</tr>
</tbody>
</table>

Specific activities are given in µMoles p-nitrophenyl phosphate hydrolyzed/hr at 37°/mg protein. The values given are the means and standard errors of the means of N separate daily preparations.
than the levels typical of most other cultured cell types.

PART 3D: PCT and PCF cells are resistant to infection by the mouse parvovirus, MVM

As discussed above, Swartzendruber and Lehman (1975) and Lehman et al. (1975) have shown that embryonal carcinoma cells are resistant to infection by SV40 and polyoma, two small DNA oncogenic viruses. These viruses were, however, able to infect differentiated (non-pluripotent) teratocarcinoma-derived cells. Stimulated by their findings, I investigated the interactions of a mouse parvovirus, MVM, with ECC cultures and other cell lines. In a collaborative study (Miller, Ward, and Ruddle, submitted for publication), I established that resistance to MVM infection was characteristic of ECCs in culture, but that teratoma-derived fibroblasts, like most non-teratoma lines examined, were susceptible to MVM infection. The immunofluorescent assay for MVM antigens is illustrated in figure 3-9, which shows that PCC4azal, unlike A9 (an established mouse fibroblast line), TDla (a teratocarcinoma-derived fibroblast), and FBU, fails to produce detectable nuclear antigen upon exposure to MVM virus. Table 3-5 summarizes the results of a similar set of experiments in which the effect of increased viral MOI was quantitated.

PCT and PCF hybrid cells, like their PCC4azal parent, are resistant to MVM infection. Each PCT clone was tested in three separate experiments, and each PCF clone tested 2-5 times. Representative areas of infected and stained cell cultures are shown in figures 3-9e,f. No immunofluorescent nuclei were seen in any of the experiments, except
Figure 3-9

Immunofluorescent detection of MVM intranuclear antigen in PCT and PCF hybrid cells, and in controls.

a) A9 mouse fibroblast; positive control.
b) TDla; teratocarcinoma-derived fibroblast.
c) PCC4azal; embryonal carcinoma parent.
d) FBU; Friend erythroleukemia cell parent.
e) PCTA1.
f) PCFB2.

Note that all the cells have a dim cytoplasmic fluorescence (dull red under the microscope) due to the Evans Blue counterstain. Some A9, TDla, and FBU cells have, in addition, a bright nuclear fluorescence (green in the original) due to the presence of MVM viral antigen. PCT and PCF cells, like their PCC4azal parent, do not contain MVM antigen after exposure to virus.
**TABLE 3-5**

**MVM INFECTION: PROPORTION OF STAINED CELLS**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>MOI</th>
<th>p</th>
<th>S.D.</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>A9</td>
<td>10</td>
<td>.22</td>
<td>.019</td>
<td>501</td>
</tr>
<tr>
<td>A9</td>
<td>200</td>
<td>.35</td>
<td>.027</td>
<td>307</td>
</tr>
<tr>
<td>PCC4azal</td>
<td>10</td>
<td>.00033</td>
<td>.00033</td>
<td>3075</td>
</tr>
<tr>
<td>PCC4azal</td>
<td>200</td>
<td>.0018</td>
<td>.0005</td>
<td>7380</td>
</tr>
<tr>
<td>TSD4</td>
<td>10</td>
<td>.37</td>
<td>.026</td>
<td>341</td>
</tr>
<tr>
<td>FBU</td>
<td>10</td>
<td>.033</td>
<td>.006</td>
<td>947</td>
</tr>
</tbody>
</table>

MOI gives the number of PFU of virus added per cell, assuming that one population doubling has occurred between subculture and addition of virus. Column p gives the ratio of immunofluorescent cells to total cells examined, and S.D. gives the standard deviation of this number. N is the actual number of cells counted for A9, FBU, and TSD4, and the approximate number scanned for PCC4azal, estimated by counting the cell number in the first five fields examined, and multiplying by the number of fields looked at in all.
that two of the PCT clones, in one of three experiments conducted, had areas with several positive nuclei. Each experiment involved the examination of several thousand cells from each hybrid clone.

MVM is known to require S-phase cells for its proliferation (Tattersall, 1972). There was no indication that my conditions of infection interfered with the cell cycle in the resistant cultures, but, in order to eliminate this consideration entirely, I tested infected cultures autoradiographically for the ability to incorporate thymidine into cell nuclei. Virus-treated hybrid cells, like PCC4azal cells, were able to accumulate intranuclear \(^3\)H-thymidine but did not accumulate viral antigen; A9 cultures tested in parallel had many nuclei which were both radioactive and fluorescent.

As shown above, FBU cells are susceptible to MVM infection, and this susceptibility is extinguished in the PCF hybrids. It is not meaningful to test mouse thymocytes in the same way, as a control for the PCT hybrids, since thymus cells do not divide in culture, while MVM infects only proliferating cells. Concanavalin-A-stimulated splenic lymphoid cells, most of which are derived from thymus-processed lymphocytes (Stobo et al., 1972), were also tested, and were found not to accumulate MVM antigen. This is not surprising, considering that MVM, like other paroviruses, seems not to interfere with mitotic cell populations in adult animals (for review, see Toolan, 1972).

In summary: PCT and PCF hybrids resemble ECCs in that all three cell types, unlike most permanent mouse cell lines, are resistant to infection by the parovirus MVM. Whatever factors make Friend cells susceptible to MVM infection are extinguished after hybridization with the ECC line, PCC4azal.
PART 3E: PCT and PCF cells give rise to multidifferentiated tumors, some of which produce α-fetoprotein.

Each clone of PCT and PCF hybrid cells was tested to see whether it could produce tumors after subcutaneous inoculation into mice. Even after inoculation of as many as $10^7$ cells into genetically appropriate animals, "take" incidence of some hybrid lines remained low, as is shown by the data presented in Table 3-6. A detailed study of those factors (age and sex of host, size of inoculum, syngeneic vs. nude host, etc.) which might influence the fraction of successful inoculations was not carried out.

Tumors were, however, produced from all of the four PCT clones and from four of the five PCF clones. Some tumors were produced in mice homozygous for the gene "nude," which impairs cell-mediated immune responses (Freedman and Shin, 1974); others were produced in syngeneic $F_1$ hybrid mice. Of the three PCT tumors tested, all three were shown to be transplantable: pieces of tumor tissue removed at autopsy and inserted subcutaneously into $F_1$ mice gave rise to progressively growing tumors.

Cell lines were derived from four of the PCT tumors and tested for chromosome and isozyme content to see to what extent the tumor cells resembled the originally inoculated population. As shown in Table 3-7, all four tumor-derived cell lines retained GPI heterodimer, and thus are known to contain at least one chromosome from each of the parental cell strains. Three of the four tumor lines had chromosome numbers indistinguishable from those of the original populations; the other had lost about 7 of its original 75 chromosomes. By the end of their
TABLE 3-6

INCIDENCE OF TUMOR FORMATION BY PCT AND PCF HYBRID CELLS

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCTA1</td>
<td>4/4</td>
</tr>
<tr>
<td>PCTA2</td>
<td>4/6</td>
</tr>
<tr>
<td>PCTBla</td>
<td>3/4</td>
</tr>
<tr>
<td>PCTB1b</td>
<td>4/9</td>
</tr>
<tr>
<td>Total PCT</td>
<td>15/23 (65%)</td>
</tr>
<tr>
<td>PCFA8</td>
<td>3/7</td>
</tr>
<tr>
<td>PCFB1</td>
<td>0/4</td>
</tr>
<tr>
<td>PCFB2</td>
<td>1/4</td>
</tr>
<tr>
<td>PCFB3</td>
<td>2/4</td>
</tr>
<tr>
<td>PCFB5</td>
<td>2/4</td>
</tr>
<tr>
<td>Total PCF</td>
<td>8/23 (35%)</td>
</tr>
</tbody>
</table>

Tumor incidence is expressed as number of tumors/number of animals inoculated. Three tumors produced by transplantation of PCTB1b primary tumors have been omitted from the data in this table.
TABLE 3-7
CHROMOSOME NUMBER AND ISOZYME ANALYSES OF CELL LINES DERIVED FROM PCT TUMORS

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Cell Line</th>
<th>Mean #</th>
<th>S.D.</th>
<th>N</th>
<th>Orig. #</th>
<th>GPI Dimer</th>
</tr>
</thead>
<tbody>
<tr>
<td>T11-2</td>
<td>PCTA2</td>
<td>72</td>
<td>4.6</td>
<td>21</td>
<td>73</td>
<td>+</td>
</tr>
<tr>
<td>T11-3</td>
<td>PCTA2</td>
<td>72</td>
<td>2.6</td>
<td>19</td>
<td>73</td>
<td>+</td>
</tr>
<tr>
<td>T4-3</td>
<td>PCTB1a</td>
<td>68</td>
<td>3.0</td>
<td>19</td>
<td>75</td>
<td>+</td>
</tr>
<tr>
<td>943</td>
<td>PCTB1b</td>
<td>75</td>
<td>2.3</td>
<td>21</td>
<td>74</td>
<td>+</td>
</tr>
</tbody>
</table>

Cultured cells derived from tumors of the indicated hybrid cell lines were used to prepare metaphase spreads 3-4 subculturings after initiation of each culture. N metaphases were counted to yield a mean chromosome number and a standard deviation (S.D.) for each distribution. The mean chromosome number characteristic of the inoculated cell line is also listed. The final column indicates the GPI heterodimer was demonstrated in each tumor-derived population.

Analogous data for PCF-derived tumors is not available.
second passage in vitro, these tumor-derived cell lines contained only cells which resembled ECCs (and hence the inoculated PCT cells) morphologically.

All the tumors were fixed, sectioned, and stained with hematoxylin and eosin for histological evaluation. Table 3-8 presents the results of the histological analysis of the PCT tumors. Illustrative histological sections are presented in figures 3-10, 3-11, and 3-12, as well as in 3-14c,d. Because of the lower "take" incidence of the PCF cell lines (see Table 3-6), fewer PCF-derived tumors were available for analysis; Table 3-9 summarizes the data from the eight PCF tumors obtained. Figures 3-13 and 3-14a,b show sections of PCF tumors.

All of the tumors contained embryonal carcinoma, examples of which are shown in figures 3-10a and 3-13a. The presence of embryonal carcinoma in hybrid-derived tumors is consistent with the other data suggesting that these cell lines resemble their ECC parent, PCC4azal. The three PCFA8 tumors were composed entirely of embryonal carcinoma, and several of the PCT tumors were largely embryonal carcinoma, in which could be found small islands of differentiated tissue.

All the PCT tumors, and each of the five PCFB2, PCFB3, and PCFB5 tumors contained at least some differentiated tissue. In general, tumors with the greatest variety of tissue types also contained the largest proportion of non-ECCs. Several of the tumors were largely necrotic at the time of autopsy, but contained nodules of cartilage; these tumors are left otherwise undiagnosed (empty spaces) in Table 3-8.

Some tissue types were seen frequently. All the tumors (except, of course, those from PCFA8) had at least some primitive neural tissue,
TABLE 3-8

DIFFERENTIATED TISSUES FOUND IN PCT HYBRID TUMORS

<table>
<thead>
<tr>
<th>Tissue</th>
<th>PCTA1</th>
<th>PCTA2</th>
<th>PCTBla</th>
<th>PCTBlb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryonal carcinoma</td>
<td>+ + +</td>
<td>+ +</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>Ectodermal cords</td>
<td>- + +</td>
<td>+ +</td>
<td>+ + +</td>
<td>- + +</td>
</tr>
<tr>
<td>Primitive neural tissue</td>
<td>+ + +</td>
<td>+ +</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>Mature neural tissue</td>
<td>- + +</td>
<td>+ +</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>Stratified squamous epithelium</td>
<td>- - -</td>
<td>- +</td>
<td>- - -</td>
<td>- + +</td>
</tr>
<tr>
<td>Mesenchyme</td>
<td>- + +</td>
<td>- -</td>
<td>- - -</td>
<td>- + -</td>
</tr>
<tr>
<td>Muscle</td>
<td>- + +</td>
<td>- -</td>
<td>- - -</td>
<td>- + -</td>
</tr>
<tr>
<td>Cartilage</td>
<td>- + +</td>
<td>+ - +</td>
<td>+ - +</td>
<td>- - +</td>
</tr>
<tr>
<td>Endodermal epithelum</td>
<td>+ + +</td>
<td>+ +</td>
<td>- - -</td>
<td>+ + +</td>
</tr>
<tr>
<td>Ciliated epithelum</td>
<td>- + +</td>
<td>- -</td>
<td>- - -</td>
<td>- + +</td>
</tr>
<tr>
<td>Glandular structures</td>
<td>- + +</td>
<td>- -</td>
<td>- - -</td>
<td>- + +</td>
</tr>
<tr>
<td>Parietal yolk sac</td>
<td>- + +</td>
<td>- -</td>
<td>- - -</td>
<td>- - -</td>
</tr>
<tr>
<td>Polyploid giant cells</td>
<td>- + +</td>
<td>- -</td>
<td>- - -</td>
<td>- - +</td>
</tr>
<tr>
<td>α-fetoprotein</td>
<td>+ - -</td>
<td>- - -</td>
<td>- - -</td>
<td>- + +</td>
</tr>
</tbody>
</table>

A (+) sign indicates that the tissue was found in the tumor indicated; a (-) sign indicates absence. An empty space in the α-fetoprotein row shows that no test was carried out for the animal in question. Tumors 954 and 973 were largely necrotic, but nodules of cartilage were apparent in each. All tumors are primary, except that T4-3, T4-4, and T4-5 were derived from transplantation of T4-2; T4-5 is a second generation transplant. T-series tumors were produced in syngeneic (C3H/HeJ x 129/Sv)F1 mice, and the others in nu/nu animals.
Figure 3-10

Hematoxylin- and eosin-stained sections of PCT hybrid tumors.

a) Typical poorly differentiated area, with an ectodermal cord at center left. Tumor 876; x160.

b) Well differentiated area. Note ciliated epithelium (ce), cartilage (ca), as well as embryonal carcinoma (ec). Tumor T8-1; x180.

c) Well differentiated area. Note glandular tissue (gl) and several endodermal vesicles (for example, en). Neural tubule at left of center. Tumor T8-1; x180.

d) Ectodermal cords. Tumor T4-4; x180.
Figure 3-11

Hematoxylin- and eosin-stained sections of PCT hybrid tumors.

a) Endodermal vesicle. Tumor 943; x145.

b) Ciliated epithelium. Tumor T8-1; x440.

c) Parietal yolk sac. Note Reichert's membrane indicated by arrows. Tumor T8-1; x440.

d) Glandular tissue. Tumor T8-1; x180.
Figure 3-12

Hematoxylin- and eosin-stained sections of PCT hybrid tumors.

a) Cartilage. Tumor T4-1; x180.
b) Immature muscle. Tumor T4-1; x440.
c) Neural tubules. Tumor 943; x180.
d) Giant cells. Tumor T8-1; x440.
### TABLE 3-9

DIFFERENTIATED TISSUES FOUND IN PCF HYBRID TUMORS

<table>
<thead>
<tr>
<th>Tissue</th>
<th>PCFA8</th>
<th>PCFB1</th>
<th>PCFB2</th>
<th>PCFB3</th>
<th>PCFB5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryonal carcinoma</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ectodermal cords</td>
<td>---</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Primitive neural tissue</td>
<td>---</td>
<td>+</td>
<td>++</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Mature neural tissue</td>
<td>---</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Cartilage</td>
<td>---</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Muscle</td>
<td>---</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Endodermal epithelium</td>
<td>---</td>
<td>-</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ciliated epithelium</td>
<td>---</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

α-fetoprotein                  | ---   | -     | ---   | +     |

A (+) sign indicates that the tissue was present in the tumor; a (-) sign denotes absence. No test for α-fetoprotein was performed on animal T3-2. Tumors 1042 and 1043 were produced in nu/nu mice, and all others in syngeneic (DBA/2J x 129/Sv)F$_1$ animals.
Figure 3-13

Hematoxylin- and eosin-stained sections of PCF hybrid tumors.

a) Embryonal carcinoma cells. T7-1; x175.

b) Cavities lined with ectodermal epithelium, with embryonal carcinoma at lower left and primitive neural tissue in the central band. T3-1; x175.

c) Cavities lined with ciliated endodermal epithelium.
   T13-1; x430.

d) Primitive neural tissue with organized neural tubules.
   T13-1; x430.
Figure 3-14

Hematoxylin- and eosin-stained sections of PCT and PCF hybrid tumors.

a) Rhabdomyosarcoma tissue in a PCF tumor. T13-1; x175.

b) PCF tumor with cartilage nodule, rhabdomyosarcoma tissue around it, and primitive neural tissue at right, above center. T13-1; x175.

c) Stratified squamous epithelium, with transition to ciliated epithelium, and with a vesicle of ciliated epithelium above; from a PCT tumor. T10-1; x430.

d) Mature neural tissue, from a PCT tumor. T10-1; x175.
which in some cases made up nearly all the bulk of the tumor. Organization of this tissue into neural tubules (figures 3-12c, 3-13d) was common. Mature neural tissue (figure 3-14d), cartilage nodules (figures 3-10b, 3-12a, 3-14b), cavities lined with endodermal epithelium (figure 3-11a), and cords of ectodermal epithelium (figures 3-10d, 3-13b) were present in many of the tumors. Some tissue types, including ciliated and secretory endodermal epithelium (figures 3-10b, 3-11b, 3-13c), stratified squamous epithelium (figure 3-14c), parietal yolk sac with Reichert's membrane (figure 3-11c), and glandular structures (figure 3-11d), were present only in the well-differentiated tumors. Bone and pigmented tissue were not observed; these tissues are common in primary teratomas, but considerably less so in transplantable teratocarcinomas which have been passaged for many generations (L. G. Stevens, personal communication).

Scoring for mesodermal derivatives in these tumors is difficult. Cartilage is easy to recognize, and cannot be confused with host tissue. Confident diagnosis of muscle and connective tissue is, however, tricky in poorly differentiated tumors when they are enmeshed in host connective tissue and subcutaneous muscle. As a consequence, my scores for these two sorts of tissue are most likely underestimates. I accepted mesenchyme and muscle tissue as tumor-derived only when I found them isolated from the strands of penetrating host tissue; most such areas were close to areas of cartilage. Some areas of muscle tissue (figure 3-12b) resembled immature cardiac muscle, and are thus unlikely to have been host-derived.

PCF (though not PCT) tumors contained large amounts of the tissue shown in figure 3-14a. These large, pleomorphic, multinucleated cells
were usually found near cartilage nodules, and resemble (L. C. Stevens, personal communication) cells found in rare teratocarcinomas undergoing transition into rhabdomyosarcomas. All four PCF tumors diagnosed in Table 3-9 as having muscle tissue had this unusual kind only.

Most of the tumor-bearing mice were bled just prior to sacrifice, and their sera analyzed for the presence of α-fetoprotein (AFP). Figure 3-15 presents an example of such an experiment, and the results of the analyses are given as the last lines in Tables 3-8 and 3-9. Three of the 13 PCT tumors tested, and one of 7 PCF tumors, produced detectable amounts of AFP; all four of the positive tumors were well-differentiated and contained substantial amounts of secretory endodermal epithelium. As discussed above, the best evidence suggests that AFP is not synthesized by ECCs, but rather by endodermal derivatives, including visceral yolk sac. The good correlation in these hybrid tumors between AFP secretion and the presence of endodermal derivatives adds further support to the hypothesis that these differentiated cells are responsible for AFP production in teratomas. The presence of AFP in the sera of tumor-bearing mice suggests that structural differentiation in the hybrid cells was accompanied by appropriate functional changes.

PART 3F: PCT cells lack Thy-1 (theta) antigen

Thy-1 antigen (previously called "theta") is an alloantigen first defined on thymocytes (Reif and Allen, 1964a,b), and subsequently demonstrated to be present on some brain cells, epidermal cells, thymus-processed lymphocytes (for review, see Greaves et al., 1973) and some fibroblast lines (Stern, 1973). ECCs do not have Thy-1 antigen
Figure 3-15

Discontinuous counterimmunoelectrophoretic detection of α-fetoprotein.  
Top row: rabbit antiserum to mouse α-fetoprotein, diluted 1:4.  
Bottom row: Antigen: serum or ascitic fluid from a mouse carrying the indicated tumor; diluted 1:4.

1) T8-3  
2) T7-1  
3) OTT 6050 embryoid bodies (ascitic fluid; positive control.)  
4) T3-1  
5) 971 (nude mouse carrying a fibrosarcoma hybrid tumor; negative control.)  
6) OTT 6050 embryoid bodies (serum; positive control.)  
7) T10-2  
8) T13-2  
9) T13-1

Wells 3, 4, 6, and 7 are positive for α-fetoprotein.
detectable by immunofluorescence (Stern et al., 1975). As mentioned
above, Thy-1 antigen has been shown to be extinguished when normal
thymocytes (Parkman and Merler, 1973) or thymic leukemia cells (Liang
and Cohen, 1975) have been hybridized with Thy-1^ fibroblasts.

PCT hybrid cells were examined for Thy-1 alloantigen by a cyto-
toxic absorption test. Since both 129/Sv and C3H/HeJ animals carry
the Thy-1^ allele, which produces Thy-1.2 antigen, an AKR/J-anti-
C3H/HeJ thymocyte serum, produced and titred as described above, was
used in a cytotoxic absorption test (see Part 2K) using C3H/HeJ
thymocytes as target cells. Each PCT clone was tested 5 times; Table
3-10 shows the result of a representative experiment.

Each aliquot of absorbed serum was divided into two portions,
which were then tested independently for cytotoxic activity. In the
experiment presented in Table 3-10, the two portions of AKR/J-thymocyte-
absorbed serum killed 79% and 80% of the 100 cells counted. The table
records the mean of these "% killed" scores, as well as the standard
error of the mean. The cytotoxic index, calculated as described in
Part 2K above, shows that AKR-absorbed serum killed 79% of the
thymocytes excluding those killed by C' alone; the value of 90% residual
cytotoxicity indicates that this AKR-absorbed serum had a cytotoxic
index 90% of that for unabsorbed serum tested on the same day.

The table shows that (C3H/HeJ x 129/Sv)^F_1 mouse thymocytes, as
expected, removed all of the Thy-1 cytotoxic activity from the serum.
Three of the PCT hybrid clones removed about as much activity as did
AKR thymocytes; the fourth clone, PCTBl a, removed somewhat more in this
particular test, though not in replicate experiments. All four PCT
clones repeatedly removed small amounts of cytotoxic activity, usually
TABLE 3-10

CYTOTOXIC ABSORPTION TEST FOR THY-1.2 ANTIGEN

<table>
<thead>
<tr>
<th>Absorbed With</th>
<th>Mean % Killed</th>
<th>S.E.M.</th>
<th>Cytotoxic Index</th>
<th>Residual Cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKR/J thymocytes</td>
<td>79.5</td>
<td>0.5</td>
<td>79</td>
<td>90</td>
</tr>
<tr>
<td>C12F1 thymocytes</td>
<td>4.5</td>
<td>0.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PCTA1</td>
<td>77.0</td>
<td>3.0</td>
<td>76</td>
<td>86</td>
</tr>
<tr>
<td>PCTA2</td>
<td>80.0</td>
<td>3.0</td>
<td>79</td>
<td>90</td>
</tr>
<tr>
<td>PCTBla</td>
<td>54.5</td>
<td>5.5</td>
<td>52</td>
<td>59</td>
</tr>
<tr>
<td>PCTB1b</td>
<td>80.5</td>
<td>1.5</td>
<td>80</td>
<td>91</td>
</tr>
<tr>
<td>Unabsorbed serum</td>
<td>89.0</td>
<td>1.0</td>
<td>88</td>
<td>(100)</td>
</tr>
<tr>
<td>No serum; C' only</td>
<td>4.5</td>
<td>2.5</td>
<td>(0)</td>
<td>—</td>
</tr>
</tbody>
</table>

Two volumes of serum diluted 1:1600 were absorbed with one volume of packed cells. Further experimental details are given in the text. "Values in parentheses are assigned by definition.

C12F1 = (C3H/HeJ x 129/Sv)F1.
to about the same extent as did AKR/J thymocytes. Since AKR/J mice
carry the Thy-1\textsuperscript{a} allele, and are hence unable to produce Thy-1.2
antigen, the small amount of antibody they absorb from the serum is
likely unrelated to the Thy-1 system. (The antiserum was not produced
in congenic mice, and thus may very well include antibodies specific
for other antigens.) It is possible that the PCT cells exhibit a very
small amount of Thy-1.2 antigen, or, more likely, that they absorb
small amounts of the other specificities detected by the AKR-anti-C3H
serum. They clearly produce much less Thy-1 antigen than thymus cells
from hybrid mice.

Dr. K. Kano (S.U.N.Y., Buffalo, N.Y.) has also tested these cells,
in his laboratory and using different antisera, for the presence of
Thy-1.2 antigen. Neither PCC4azal nor any of the four PCT clones had
Thy-1 antigen detectable either by absorption or mixed hemagglutination
tests.

PART 3G: PCT cells exhibit H-2 antigens

Although most mouse cells, including thymocytes, exhibit H-2
antigens on their surface (for review, see Klein, 1975), embryonal
carcinoma cells are said not to; antibody-mediated cytotoxic tests
(Artzt and Jacob, 1974) and immunofluorescence (Stern et al., 1975)
have both been shown not to detect H-2 on ECCs. The PCT hybrid clones
and their PCC4azal parent were therefore tested to see whether they
could absorb cytotoxic anti-H-2 antibodies. Since the parental genomes
differed at the H-2 loci (129/Sv carries haplotype bc, which is serol-
ogically indistinguishable from b, and C3H/HeJ carries haplotype k),
it was possible to look for the independent expression of H-2 genes from either genome. The antisera used and the procedure employed have been discussed above; as summarized in Table 2-1, the test for H-2$^{bc}$ utilizes a serum directed against the private specificity, 33, shared by the K$^b$ and K$^{bc}$ antigens (as well as against several public specificities not present on K$^k$ or D$^k$), while the anti-H-2$^k$ test employs a serum which will react against the private specificity, 23, present on the K$^k$ molecule, and against several public specificities.

Table 3-11 presents the results of several experiments in which the anti-H-2$^k$ serum was absorbed with 0.5 volumes of packed PCT (or control) cells, and then split into two portions which were tested on B10.BR mesenteric lymph node cells for residual cytotoxic activity. Unabsorbed serum, by definition, had a residual cytotoxicity of 100. Serum absorbed with A9 cells (transformed C3H/HeJ fibroblasts; H-2$^k$) had a residual cytotoxicity of 6 (data not shown). As expected, PCC4azal and F9 cells do not absorb H-2$^k$ antibodies, since these cells do not carry this haplotype. All four PCT hybrid cell lines absorb significant amounts of anti-H-2$^k$ cytotoxic activity. The rightmost column of Table 3-11 shows the result of a statistical test of the hypothesis that the cell line in question absorbs the same amount of cytotoxic activity as PCC4azal. As evaluated by Student's t-test (Snedecor and Cochran, 1967), all four PCT cell lines differ from PCC4azal in this respect, at the p<.01 significance level. Furthermore, although the 95% confidence intervals for mean residual cytotoxicity include 100 for F9- and PCC4azal-absorbed serum, the intervals for three of the four PCT hybrid lines do not include 100; this data is also included in Table 3-11. (The first of these tests is the more
TABLE 3-11

H-2\(^k\) ANTIGEN IN PCT HYBRID CELLS AND CONTROLS

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Mean RC</th>
<th>S.E.M.</th>
<th>95% Limits</th>
<th>N</th>
<th>t-statistic (vs. PCC4azal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCC4azal</td>
<td>107</td>
<td>7</td>
<td>77-137</td>
<td>3</td>
<td>---</td>
</tr>
<tr>
<td>F9</td>
<td>109</td>
<td>14</td>
<td>49-169</td>
<td>3</td>
<td>.12</td>
</tr>
<tr>
<td>PCTA1</td>
<td>17</td>
<td>7</td>
<td>0-47</td>
<td>3</td>
<td>9.14**</td>
</tr>
<tr>
<td>PCTA2</td>
<td>27</td>
<td>7</td>
<td>0-57</td>
<td>3</td>
<td>8.28**</td>
</tr>
<tr>
<td>PCTBlb</td>
<td>20</td>
<td>10</td>
<td>0-147</td>
<td>2</td>
<td>7.86**</td>
</tr>
<tr>
<td>PCTBlb</td>
<td>3</td>
<td>4</td>
<td>0-20</td>
<td>3</td>
<td>13.56**</td>
</tr>
</tbody>
</table>

For each cell line, the results of N separate assays (each involving duplicate determinations) were averaged to give a mean residual cytotoxicity (Mean RC), which is a cytotoxic index expressed as a percentage of that found for unabsorbed serum in the same test. S.E.M. gives the standard error of this mean, and the limits of the 95% confidence interval for the mean are also given. The rightmost column shows the t-statistic computed to test the hypothesis that the cell line in question differs from PCC4azal in Mean RC. (**) indicates that all four PCT clones differ from PCC4azal in this respect at the p<.01 confidence level.
convincing, since the absorption of small but significant amounts of activity could be ascribed to serum dilution by diluent trapped in the cell pellet, or to non-specific binding; but these effects, if present, are likely to have affected negative control cells as well.) On average, the unabsorbed antiserum, at the dilution used, killed about 70% of the target cells in these tests; PCT absorbed sera, on average, killed about 20-30% of the B10.BR targets. When these values are compared to the titration curve for D3b serum (not shown), one finds that PCT absorption removes between 50-70% of the cytotoxic antibody. Since the largest portion of antibody in D3b serum seems likely to be directed against classical H-2 antigens (and not, for example, Ia antigens), we can conclude that PCT cells probably exhibit \( K^k \).

The expression of H-2\(^k\) antigens on the surface of cells of all four PCT clones has been confirmed by K. Kano using a mixed hemagglutination technique (K. Kano, personal communication).

Table 3-12 presents the results of similar tests for H-2\(^{bc}\) antigen. Three control cell lines were tested: A9 and FBU do not carry the genes for H-2\(^{bc}\) antigens, and F9, though from a 129/Sv mouse, has been shown not to express H-2 antigens (Artzt and Jacob, 1974). As expected, the 95% confidence interval for the mean residual cytotoxicity of each line includes 100; F9-absorbed serum, in three tests, retained 95.7% (±2.8% standard error of the mean) of the cytotoxic activity present in unabsorbed serum tested at the same time.

PCC4azal, and three of the PCT hybrid clones, absorbed statistically significant (though very small) amounts of cytotoxic activity from D33 antiserum tested on C57BL/10 mesenteric node lymphocytes; the 95% intervals for these three cell lines do not include 100. PCTA1 and
TABLE 3-12

H-2^b ANTIGEN IN PCT HYBRID CELLS AND CONTROLS

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Mean RC</th>
<th>S.E.M.</th>
<th>95% Limits</th>
<th>N</th>
<th>t-statistic (vs. F9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A9</td>
<td>103</td>
<td>3.5</td>
<td>59-147</td>
<td>2</td>
<td>---</td>
</tr>
<tr>
<td>FBU</td>
<td>98</td>
<td>6.6</td>
<td>77-119</td>
<td>4</td>
<td>---</td>
</tr>
<tr>
<td>F9</td>
<td>96</td>
<td>2.8</td>
<td>84-108</td>
<td>3</td>
<td>---</td>
</tr>
<tr>
<td>PCC4azal</td>
<td>83</td>
<td>4.9</td>
<td>69-97</td>
<td>5</td>
<td>1.81</td>
</tr>
<tr>
<td>PCTA1</td>
<td>71</td>
<td>3.4</td>
<td>56-86</td>
<td>3</td>
<td>5.50**</td>
</tr>
<tr>
<td>PCTA2</td>
<td>76</td>
<td>8.3</td>
<td>40-112</td>
<td>3</td>
<td>2.18</td>
</tr>
<tr>
<td>PCTB1a</td>
<td>76</td>
<td>5.0</td>
<td>60-92</td>
<td>4</td>
<td>3.17*</td>
</tr>
<tr>
<td>PCTB1b</td>
<td>84</td>
<td>4.6</td>
<td>69-99</td>
<td>4</td>
<td>1.95</td>
</tr>
</tbody>
</table>

For each cell line, the results of N separate assays (each involving duplicate determinations) were averaged to give a mean residual cytotoxicity (Mean RC), which is a cytotoxic index expressed as a percentage of that found for unabsorbed serum in the same test. S.E.M. gives the standard error of this mean, and the limits of the 95% confidence interval for the mean are also given. The rightmost column shows the t-statistic computed to test the hypothesis that the cell line in question differs from F9 in Mean RC. (***) indicates significance at the p<0.01 level; (*), at the p<0.05 level.
PCTBla absorbed more antiserum than negative control F9 cells, as tested by Student's t-test, at the levels of significance indicated in the table. The other two PCT lines and PCC4azal may have absorbed more antibody than F9 cells, but not significantly more.

When changes in the proportions of target cells killed are compared with the titration curve for this cytotoxic system, it is clear that absorption with PCT or PCC4azal cells removes less than half of the serum's cytotoxic activity. It seems possible, therefore, that the antibodies removed by these cells could be "contaminating" molecules not directed against the classical H-2 antigens themselves. From this perspective, what seems to be low-level expression of H-2\textsuperscript{bc} might reflect the limitations in specificity of our cytotoxic system.

PCT and PCC4azal cells are pluripotent; PCT cultures, especially, sometimes contain small clusters of morphologically differentiated cells. It is possible that a small proportion of the cells in the cultured populations exhibit H-2\textsuperscript{bc}, but that the majority of cells lack this antigen. Small amounts of H-2\textsuperscript{bc} have been demonstrated on PCC4 cells under some culture conditions (K. Artzt, personal communication). Heterogeneity of antigen expression in these cells could be evaluated by immunofluorescence.

PCT cells absorb more cytotoxic activity from the anti-H-2\textsuperscript{k} serum used than from the anti-H-2\textsuperscript{b} serum. Although it is possible that these cells actually express more H-2\textsuperscript{k} product (from the thymocyte genome) than H-2\textsuperscript{bc} (from the teratocarcinoma genome), the tests presented in this thesis do not prove this. The two sera may, for example, differ in a) the number of different antigenic determinants recognized; b) the concentrations of antibody specific for each determinant; and c) the distribution of cytotoxic activity among the individual antibody species.
It is also possible that the two target cell populations might differ in susceptibility to C'. It is thus impossible to infer a simple relation between mean residual cytotoxicity values and numbers of H-2 molecules produced by the absorbing cell.

Two of the PCF lines (PCFA8 and PCFB2) absorb significant amounts of anti-H-2^b activity as well, but these lines have not been studied as extensively, and the data will not be presented here.

PART 3H: PCF cells resemble PCC4azal enzymatically

FBU and PCC4azal cells differ in their levels of two enzymes (besides alkaline phosphatase, which has been discussed above): FBU cells have high levels of acetylcholinesterase (E.C. 3.1.1.7; AChE), and PCC4azal cells express the "B" allele of lactate dehydrogenase (E.C. 1.1.1.27; LDH), as well as the LDH-A allele (which both parents express). All five PCF clones resemble PCC4azal in both respects.

Auerbach and Brinster (1967) have reported that pre-implantation mouse embryos, from the oocyte stage to that of the 5th-day blastocyst, contain LDH-1 (i.e., only B subunits) to the virtual exclusion of the other LDH isozymes (which contain some of the A subunit). Shortly after implantation, however, the LDH pattern changes radically, so that by day 7, and until day 12, 90% of the LDH in the embryo and its protective membranes is LDH-5, which contains only A subunits. When implantation is delayed hormonally, the changeover in LDH pattern was similarly delayed, although the blastocysts were not otherwise damaged and retained the ability to implant when the hormonal treatment was over. This result suggests that the synthesis of LDH-A subunits is linked to
implantation per se.

As shown in figure 3-16, PCC4azal cells synthesize both LDH-A and LDH-B, and, in this respect, resemble neither cells of pre-implantation embryos nor of the early post-implantation stages. The LDH pattern of embryonal carcinoma cells other than those long since adapted to culture has not, to my knowledge, been reported.

FBU cells, as shown in figure 3-16, express only LDH-A. PCF hybrid cells, like PCC4azal, express both A and B alleles, though more A than B is produced by both the hybrid and parental cells.

Acetylcholinesterase is present in erythroid cells, including mature erythrocytes (Galehr et al., 1928) and Friend erythroleukemia cells (Conscience et al., submitted for publication). As shown in Table 3-13, PCF hybrid cells and PCC4azal ECCs contain less than 1% of the AChE activity found in the FBU parents.

PART 3I: Globin gene expression in PCF hybrid cells

Many groups (see review in Friend et al., 1974) have shown that cultures of most Friend erythroleukemic cell lines have levels of globin mRNA which, while low in comparison to the levels found in DMSO-treated cultures, are much higher than the amounts present in non-erythroid cells. Uninduced Friend cell cultures also typically contain a proportion of hemoglobinated, and hence benzidine-positive cells. This fraction varies between 0.1-5.0%; the factors responsible for the variation are not understood. FBU cultures assayed at different times and in different labs have been shown to contain between 0.0002%-1.0% benzidine-positive cells (Skoultchi, Conscience, and Fournier, personal
Figure 3-16

Lactate dehydrogenase isozymes in PCF hybrids and controls.
Lactate Dehydrogenase

(+)

(-)
### TABLE 3-13

**ACETYLCHOLINESTERASE IN PCF HYBRID CELLS AND CONTROLS**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Mean Specific Activity</th>
<th>S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>FBU</td>
<td>3900.</td>
<td>20.</td>
</tr>
<tr>
<td>PCC4azal</td>
<td>13.1</td>
<td>20.9</td>
</tr>
<tr>
<td>PCFA8</td>
<td>-1.6</td>
<td>2.1</td>
</tr>
<tr>
<td>PCFB1</td>
<td>5.3</td>
<td>1.8</td>
</tr>
<tr>
<td>PCFB2</td>
<td>26.0</td>
<td>1.0</td>
</tr>
<tr>
<td>PCFB3</td>
<td>9.1</td>
<td>2.9</td>
</tr>
<tr>
<td>PCFB5</td>
<td>6.8</td>
<td>1.3</td>
</tr>
</tbody>
</table>

Specific activities are given in pmoles acetylcholine hydrolyzed per minute at 20° per mg protein, and represent the mean of 2 assays, each done in duplicate on different days.
communication, and my own unpublished results). Uninduced FBU cultures also contain immunologically detectable hemoglobin, and globin mRNA as measured by nucleic acid hybridization (Skoultchi et al., submitted for publication).

The most sensitive of these tests for globin gene expression is the assay for globin mRNA. Preliminary results from a collaborative study (Skoultchi, Miller, and Ruddle, in preparation) show that all five PCF clones contain globin mRNA, in amounts which are much lower than those characteristic of FBU cells, but higher than the (undetectable) levels of Friend cell x fibroblast and Friend cell x hepatoma hybrids. PCC4azal cells contain little or no globin message. Growth of PCF cells in DMSO causes a small diminution of globin mRNA levels.

To rule out the suggestion that these low globin mRNA levels might be the product of a small proportion of hemoglobinated cells in an otherwise "non-erythroid" population, I have examined all five clones of PCF cells, without prior DMSO treatment, for the presence of benzidine-positive cells. In excess of $10^5$ cells of each clone were scanned, and no hemoglobinated cells were found. FBU cultures tested as positive controls were found to contain positive cells. After four days of growth in medium containing 1.5% DMSO, FBU cultures typically contain about 45% hemoglobinated cells. PCFA8 and PCFB5 cultures, on the other hand, were found not to contain any positive cells (<$10^{-5}$) after four days of growth in DMSO.

The mRNA data, together with the benzidine assays, suggest that many of the cells in each PCF clone may be expressing the globin gene at a very low level. More extensive control studies, particularly to see if globin mRNA is produced at all in PCC4azal and PCT hybrid cells,
are in progress. Regardless of their outcome, it is clear that globin gene expression in PCF hybrids is severely depressed in comparison to expression in the Friend cell parent.

PART 3J: Summary of results

I summarize here, for convenience, the properties of the PCT and PCF hybrid cell lines, and compare them with those of the parental cell lines. Table 3-14 pertains to the PCT series, and Table 3-15 to the PCF cells.
### TABLE 3-14

**SUMMARY: PROPERTIES OF PCT HYBRID CELLS AND CONTROLS**

<table>
<thead>
<tr>
<th>Property</th>
<th>PCC4azal</th>
<th>Thymocytes</th>
<th>PCT Hybrids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromosome number(^a)</td>
<td>39</td>
<td>40</td>
<td>73-75</td>
</tr>
<tr>
<td>Growth</td>
<td>attached clumps</td>
<td>suspension</td>
<td>attached clumps</td>
</tr>
<tr>
<td>Extranucleolar heterochromatin</td>
<td>very little</td>
<td>moderate</td>
<td>very little</td>
</tr>
<tr>
<td>Alkaline phosphatase(^a)</td>
<td>4 units</td>
<td>0.5 units</td>
<td>2-4 units</td>
</tr>
<tr>
<td>MVM infection</td>
<td>resistant</td>
<td>---</td>
<td>resistant</td>
</tr>
<tr>
<td>Tumors</td>
<td>teratoid</td>
<td>---</td>
<td>teratoid</td>
</tr>
<tr>
<td>Thy-1 antigen</td>
<td>no</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>H-2(^b) (note c)</td>
<td>? present</td>
<td>not present</td>
<td>present (2 clones)</td>
</tr>
<tr>
<td>H-2(^k) (note c)</td>
<td>not present</td>
<td>present</td>
<td>present</td>
</tr>
</tbody>
</table>

**Notes:**

a) Chromosome numbers and enzyme activities are means.

b) Thymocytes could not be tested for MVM susceptibility since they do not divide; mitogen-stimulated T lymphocytes are, however, resistant to MVM.

c) H-2 levels are estimated from massive absorptions, and require further exploration by single cell techniques and quantitative absorptions, as noted in the text.
### TABLE 3-15

**SUMMARY: PROPERTIES OF PCF HYBRID CELLS AND CONTROLS**

<table>
<thead>
<tr>
<th>Property</th>
<th>PCC4azal</th>
<th>FBU</th>
<th>PCF Hybrids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromosome number</td>
<td>39</td>
<td>39</td>
<td>70-73 or 94-107</td>
</tr>
<tr>
<td>Growth</td>
<td>attached clumps</td>
<td>suspension</td>
<td>attached clumps</td>
</tr>
<tr>
<td>Extranucleolar heterochromatin</td>
<td>very little</td>
<td>moderate</td>
<td>very little</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>7 units</td>
<td>&lt;0.1 unit</td>
<td>7-11 units</td>
</tr>
<tr>
<td>MVM infection</td>
<td>resistant</td>
<td>susceptible</td>
<td>resistant</td>
</tr>
<tr>
<td>Tumors</td>
<td>teratoid</td>
<td>erythroleukemia</td>
<td>teratoid</td>
</tr>
<tr>
<td>LDH isozymes</td>
<td>A and B</td>
<td>A only</td>
<td>A and B</td>
</tr>
<tr>
<td>Acetylcholinesterase</td>
<td>13 units</td>
<td>3900 units</td>
<td>0-26 units</td>
</tr>
<tr>
<td>Benzidine stain</td>
<td>negative</td>
<td>positive</td>
<td>negative</td>
</tr>
</tbody>
</table>

*Note: Chromosome numbers and enzyme activities are means.*
The results of my research, which have been summarized in Part 3J above, show that somatic cell hybrids of embryonal carcinoma cells can closely resemble their ECC parent, even to the retention of pluripotency. Yet experiments in other laboratories, especially the studies of Finch and Ephrussi (1967), Jami et al. (1973), and McBurney (1976), have shown that teratocarcinoma x fibroblast hybrids resemble fibroblasts (see Part 1G, above). How can one explain this apparent discrepancy?

Four classes of explanation come to mind. For one thing, PCT and PCF hybrid cells differ from previously studied teratocarcinoma-derived hybrids in the proportion of chromosomal material contributed to the hybrid by the teratoma and non-teratoma parents. This "gene dosage" effect on hybrid phenotype has been discussed in Part 1A, above. The three parental cell types used to produce PCT and PCF hybrids have 39 or 40 chromosomes each, with 40-43 chromosome arms. The fibroblast parents used in the three previous published reports contained 50-68 chromosomes, many of them bi-armed (in the L cell derivatives). It would be interesting in this regard to produce and test hybrids between PCC4azal and tetraploid Friend cells, or between PCC4azal and two Friend or thymus cells. As mentioned above, McBurney (1976) has shown that fusion between a tetraploid ECC and a subtetraploid 3T3 cell yields fibroblastoid hybrids.

A second possibility is that L cells and 3T3 cells, during their long history of growth in vitro, may, in the process of selection for fast-growing cells, have also acquired an unusually strong ability to
control the phenotype of somatic cell hybrids. Mere tumorigenicity is 
not sufficient to endow a cell with this (so far hypothetical) ability: 
Friend cells (including FLC, the line from which FBU was derived) are 
tumorigenic. It is possible that extensive karyotypic evolution is 
more important than tumorigenicity per se.

The discrepancy between my results and the earlier findings might 
also be due, in part, to technical considerations. It is difficult to 
tell whether the cloned, pluripotent teratocarcinoma cultures used in 
some of the previous studies may have contained "contaminating"
teratoma-derived fibroblasts, as do many such cultures (Lehman et al., 
1974; Martin and Evans, 1974). In my experience, embryonal carcinoma 
cultures have seemed to produce hybrids in low yield, relative to other 
cultured cell types, and it is possible that fibroblasts in a mixed 
teratocarcinoma culture might give rise to hybrid clones at rates 
disproportionate to their representation in the culture. Furthermore, 
the hybridizations reported by Finch and Ephrussi (1967) and by Jami 
et al. (1973) were carried out under semi-selective conditions; embry­
onal carcinoma cells were not killed, and hybrid colonies were selected 
for study because they contained fibroblastoid cells. It is possible 
that these two hybridization experiments produced pluripotent hybrids 
which were not recognized because they, like PCT and PCF cells, looked 
like their ECC parents under phase microscopy. (Similarly, it is 
possible that the PCC4azal x thymocyte fusion mixtures contained hybrid 
cells which so closely resembled thymocytes that they failed to grow 
under routine culture conditions. This possibility is in no way ruled 
out by my failure to recover proliferating suspension cells.) These 
considerations are unlikely to apply to the study of McBurney (1976),
who used ECC lines derived by a technique, cloning on feeder layers, which minimizes differentiation (Martin and Evans, 1975a), and used selective conditions which killed both parental lines.

A more intriguing explanation of the results reported in this thesis might be that differences between PCT and PCF hybrids and the teratoma-fibroblast hybrids are due not to gene dosage, or peculiarities of culture-adapted lines, or technical artifact, but rather to inherent differences between fibroblasts and Friend or thymus cells. The first step in exploring this suggestion is to ascertain whether any or all of the products of fusion between ECCs and normal diploid fibroblasts are pluripotent. If they are, then the results reported from other laboratories can be attributed to one or more of the three factors listed above. But if hybrids between ECCs and diploid fibroblasts do resemble their fibroblast parents, this will suggest that fibroblasts may differ from both Friend cells and thymocytes in the details of the mechanisms they use to maintain their respective differentiated states. This suggestion could be explored by cell fusion experiments in which fibroblasts were compared with Friend and thymus cells with respect to their aptitude for influencing hybrid phenotypes in combination with parents other than embryonal carcinoma cells.

We must also consider the idea that the PCT and PCF hybrids might resemble teratocarcinomas only because they may have lost large or particularly important parts of the genetic material contributed by the non-pluripotent parent. Several previous experiments (reviewed in Darlington and Ruddle, 1975) have suggested that retention or re-acquisition of a cell-specific characteristic may be contingent on the loss of chromosomes from the parent which did not show the trait in
question; in at least two instances (Wiener et al., 1972; Cochran et al., 1975), tumor morphology was affected in this way. It is possible that each PCT line, for example, could have lost a small number of thymocyte-derived chromosomes which, if retained, would have forced retention of thymus-specific traits, but which do not include the chromosomes coding for GPI or H-2. It is also conceivable that a large number of thymus-derived chromosomes have been lost, but have been replaced by morphologically indistinguishable PCC4azal chromosomes. These matters cannot be decided with available genetic or cytogenetic methods.

The three 2:1 PCF hybrids do lose chromosomes with continued culture; there is no evidence from these clones on the issue of preferential loss of genetic material from one parent or the other.

Cultured cell lines derived from PCT hybrid tumors show chromosome loss ranging from moderate (in one tumor, 7 of 75 chromosomes lost) to undetectable in the other three tumors examined. All four tumors retained both alleles of GPI. There is thus no evidence, at this limited resolution, for chromosome loss accompanying tumor formation in PCT hybrid cells.

My studies of the properties of PCT and PCF hybrid cells suggest a generalization: that fusions between embryonal carcinoma cells and near-diploid non-pluripotent cells produce hybrids which resemble ECCs. This hypothesis clearly requires further testing; hybrids involving other ECC lines and other differentiated cell lines should be characterized to define the limits over which this generalization applies.

Should this phenomenon prove to be general, it would suggest that ECCs, and perhaps the embryonic mesodermal precursor cells they so closely resemble, contain a molecular apparatus able to "reset" the
genome of committed and differentiated cells. Precedent for such a phenomenon can be found in the work of Gurdon and his collaborators (reviewed in Gurdon, 1974), who showed that differentiated amphibian nuclei could, if placed into an enucleated oocyte, revert to a totipotent state and carry out the normal developmental program. Similar work has been carried out by Illmensee (1973) in Drosophila. The work presented in this dissertation is not completely analogous, since the PCC4azal nuclei have not been removed, nor the Friend and thymus cell nuclei shown to be entirely reprogrammed. It is possible, for example, that the Friend and thymus cell genetic material in these hybrids, while no longer making any of the gene products characteristic of these cell types, and while still producing the constitutive enzymes and antigens I have tested for, is for some reason incapable of making ECC-specific products. Nor have Friend or thymus cell chromosomes been shown to participate directly in the developmental transitions of which these hybrids are capable. These results therefore raise a number of questions:

1) Do the thymic and erythroid genomes "convert" to pluripotency, or are they merely neutralized, expressing only those genes which are expressed in both parents?

2) If PCC4azal cells can reset other genomes, how, in molecular terms, is this accomplished? Is PCC4azal cytoplasm sufficient to reset a differentiated nucleus? Is a PCC4azal nucleus sufficient to reset the cytoplasm from some other cell type, and, if so, does this depend on the position of either parent in the cell cycle?

3) Is this reprogramming ability, hypothesized to be present in PCC4azal cells, present in all embryonal carcinoma cells, and in
embryonic mesectodermal precursor cells? If so, when is it lost: before, or after the pluripotent ECC commits itself to a particular line of differentiation?

These problems have still to be resolved. In any case, these experiments show that embryonal carcinoma cells, although nearly unrestricted in their developmental potential, can nonetheless control the pattern of gene expression in a somatic cell hybrid whose other parent is a stably differentiated cell. The ability (of ECCs) to give rise to a variety of differentiated progeny under appropriate developmental conditions does not necessarily bring with it a susceptibility to whatever factors those differentiated cells use to maintain their epigenetic state.

The work reported here bears upon three other, more specific issues as well. I want first to discuss the relevance of my work to extinction of Friend cell properties in somatic cell hybrids. As summarized above in Part 1C, the previous work on Friend cell hybrids is equally compatible with either of two hypotheses: first, that erythroid properties are extinguished whenever Friend cells are fused with non-erythroid cells; or, second, that extinction takes place whenever the non-erythroid parent contributes the majority of chromosomes to the hybrid cells. (It may well be, of course, that total chromosome number is less important than the doses of a few particular loci.) PCFB3 and PCFB5 are likely to contain about equal genetic contributions from FBU and PCC4azal, but resemble the latter parent closely. In at least this case, then, addition of but a single diploid genome from a non-erythroid cell is sufficient to extinguish the erythroid-specific properties of FBU. It is hard to predict the properties of hybrids containing twice as much
genetic material from Friend cells as from an ECC.

My results are relevant also to the question of antigen expression on ECCs. Previous studies, reviewed in Part 1F, have suggested that ECCs lack detectable H-2 antigen, but exhibit ECC-specific antigen or antigens, one of which (F9 antigen) is chemically similar to H-2 and probably coded for by an allele of the T/t complex. Ideally, one would wish to characterize the surface antigens of the PCT and PCF hybrids in a number of ways, including 1) massive absorptions to test whether the hybrid populations had any cell surface H-2 antigen; 2) fluorescent antibody tests to see whether all the cells exhibit the same antigens; 3) quantitative absorptions to see how much of each antigen was present, expressed relative to a standard absorbing cell, such as a lymph node cell from an F₁ hybrid mouse; and 4) tests for ECC-specific antigens. At present, only the first of these steps has been completed, and only for the PCT cells; conclusions about surface antigen patterns of these hybrids are therefore necessarily limited.

The data show that all four PCT hybrid populations can absorb cytotoxic activity directed against the H-2<sup>k</sup> allele contributed by the thymocyte parent. Three of the four clones, and PCC4azal cells as well, also remove cytotoxic activity from an anti-H-2<sup>b</sup> serum, and two of the PCT hybrids removed significantly more than a negative control line, F9. (H-2<sup>b</sup> is serologically indistinguishable from H-2<sup>bc</sup>, the antigen of the 129/Sv strain from which the ECC line was derived.) More cytotoxic activity is removed from the anti-H-2<sup>k</sup> serum than from the anti-H-2<sup>b</sup> serum. As discussed above (Part 3K), this result suggests that these cells may exhibit more H-2<sup>k</sup> molecules than H-2<sup>bc</sup>, but other explanations are possible and have not been ruled out.
The results suggest as well that expression of H-2 antigen is compatible with pluripotency. But another hypothesis, that some of the cells in each hybrid population have begun to express H-2 while losing pluripotency, can only be ruled out by single-cell tests for population heterogeneity.

The third specific issue I want to discuss concerns the co-ordination of properties usually expressed together on embryonal carcinoma cells. Among the many events that occur during the seventh day of mouse embryonic development, three seem especially relevant here. Ectopically transplanted embryos are able to produce teratocarcinomas only up to the seventh day of gestation; transplantation of older embryos produces only embryomas, devoid of ECCs and hence not transplantable from animal to animal (Damjanov et al., 1971c). Secondly, early embryos placed under the kidney or testis capsules develop more or less normally only up to the seventh day stage, at which point disorganization begins, culminating in teratoma formation (Stevens, 1968; 1975). Finally, this stage is also the earliest at which H-2 antigen can be detected (reviewed in Billington and Jenkinson, 1975). Although these three changes occur approximately at the same embryonic stage, it has yet to be shown that they in fact take place simultaneously. One ought, however, to consider the possibility that these transitions do occur together, and do so for a reason.

Embryonal carcinoma cells have a cluster of properties, each of which is found in few if any other cell types. As discussed in the introduction to this dissertation, ECCs are a) totipotent; b) nontumorigenic in one environment, the inner cell mass, but able to produce tumors elsewhere; c) low in H-2 antigen but high in chemically similar,
ECC-specific antigens; and d) resistant to a number of viruses to which many mouse cells are susceptible. They also have a number of properties which surprise us less (unusual chromatin distribution, characteristic enzyme activities, particular adhesive properties, etc.), but which may nonetheless have important developmental implications. Some of these properties may be contingent on others; for example, and for reasons that are not yet obvious, susceptibility to SV40 infection may be incompatible with totipotency, or with F9 antigen expression.

Somatic cell hybridization provides an opportunity to dissect such an association of properties epigenetically. The studies of Minna et al. (1972) and of Szpirer and Szpirer (1975) have provided surprising glimpses of control mechanisms in neuroblastoma and hepatoma cells, respectively. If an ECC-Friend cell hybrid, for example, were shown to be pluripotent, but yet MVM-susceptible, one could conclude that whatever metabolic specializations endowed cells with resistance to MVM were incidental, rather than fundamental, to the totipotent state.

All the PCT and PCF hybrid clones resemble embryonal carcinoma cells in each of the properties tested—enzymatic, viral, developmental, and antigenic—with the likely exception of the H-2 antigens discussed just above. The few structural differences found (e.g. nucleolar number in the hybrids is usually greater than in the PCC4azal parents, and cell size is also somewhat larger) may be due simply to the greater ploidy of the hybrids; nucleolar number of tetraploid, pluripotent ECCs has not, to my knowledge, been reported. The marginally higher content of globin mRNA sequences in PCF cells, as compared to other Friend cell hybrids and to PCC4azal controls, may be a more significant exception, and is still under investigation (Skoultchi, Miller, and Ruddle, in
preparation). Continued association of all these ECC properties in
two kinds of hybrids clearly does not prove that any of the traits
depends on any of the others, much less reveal the nature of the link.
Dissociation may be brought about in other hybrid combinations, or in
some other kind of experiment altogether. The most one can say is
that the intertypic hybridizations reported here provide no evidence
that any of the ECC-specific properties can be expressed (or extin-
guished) independently of the whole set.

This discussion has encompassed a fair number of still-unanswered
questions. I wish to close by mentioning one other avenue for future
exploration. It will soon be routine to transfer portions of the
human genome, ranging in size from the entire diploid set, to individual
chromosomes, to small sections of chromosomes, and at high efficiency,
into any mouse cell which lacks HGPRT. The results presented in this
dissertation make it seem likely that at least some of the resultant
partial hybrids will be pluripotent. It is likely, too, that these
cells will produce the enzymes, antigens (including human "T/t" anti-
gens), etc., characteristic of the early and inaccessible stages of
human embryogenesis, and hence not easy to obtain in other ways.
Furthermore, it is not at all impossible that these partial hybrids, if
injected into the blastocyst cavity of early mouse embryos, will par-
ticipate in the formation of a mosaic mouse. It would be interesting,
I think, to see how the human genes which mediate such embryonic
decisions as growth rate, size, germ layer orientation, etc., in which
mouse and human embryos differ, would interact with the intact mouse
genome in these "embryogenic" hybrid cells.
LITERATURE CITED


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