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Genetics

Genetic analysis of indefinite division in human cells: Identification of four complementation groups

(cellular aging/immortalization)

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ABSTRACT **Hybrids obtained following fusion of normal human diploid fibroblasts with different immortal human cell lines exhibited limited division potential. This led to the conclusion that the phenotype of cellular senescence is dominant and that immortal cells arise as a result of recessive changes in the growth control mechanisms of the normal cell. We have exploited the fact that immortality is recessive and, by fusing immortal human cell lines with each other, assigned 21 cell lines to at least four complementation groups for indefinite division. A wide variety of cell lines was included in the study to determine what parameters, if any, would affect complementation group assignment. The results indicate that cell type, embryonal layer of origin, and type of tumor do not affect group assignment. There does not appear to be any correlation between expression of an activated oncogene and group assignment. However, all of the immortal simian virus 40-transformed cell lines studied (with the exception of one xeroderma-pigmentosum fibroblast-derived line) assign to the same group, indicating that this virus immortalizes various human cells by the same processes. The assignment of immortal human cells to distinct groups provides the basis for a focused approach to determine the genes important in normal growth regulation that have been modified in immortal cells.**

The limited division potential of normal human cells in culture is well documented and accepted as a model for aging at the cellular level (1, 2). However, despite the importance to many fields, including neoplasia and abnormal development, very little is known about the mechanisms that limit normal cell division potential and those that permit tumor-derived or virus- or carcinogen-treated cells to proliferate indefinitely (i.e., become immortal). A number of hypotheses have been proposed to explain the phenomenon of cellular senescence, and they can be grouped into two broad categories. One set proposes that the loss of proliferative potential is due to random accumulation of damage, such as mutations or errors in protein and RNA synthesis (3, 4). The other set proposes that genetically programed processes, such as those observed in differentiation, result in cell aging (5–7). We and others have found that hybrids obtained from fusion of normal cells with immortal cells exhibit limited division potential (8–11). These results indicated that the phenotype of cellular senescence was dominant and that immortality resulted from recessive changes in normal growth regulatory genes. They also supported the hypothesis that cellular senescence was a genetically programed process rather than the result of random accumulation of damage. This hypothesis was further strengthened when we demonstrated, in a limited study, that fusions among some immortal human cells yielded hybrids having limited division potential (11). This result could be explained on the basis of complementation of different recessive changes that had led to immortalization in the parent cell lines fused, to yield a hybrid in which all normal growth regulatory genes were now present and expressed. We have extended these studies to include and assign 21 different immortal human cell lines to complementation groups for indefinite division. The purpose of the study was 2-fold: (*i*) to determine the degree of complexity of the processes limiting normal cell growth by the number of complementation groups identified and (*ii*) to identify any parameters that might affect complementation group assignment by studying a wide variety of cell lines.

MATERIALS AND METHODS

Hybrid isolation involved the use of "universal hybridizer" cell lines—i.e., cell lines having a dominant and recessive marker. The dominant markers were ouabain or neomycin resistance and the recessive markers were deficiency in either the enzyme hypoxanthine guanine phosphoribosyl transferase or thymidine kinase. Following fusion with any wild-type human cell, selection of hybrids was achieved by use of medium containing hypoxanthine, aminopterin, and thymidine (12) and ouabain or the drug G418. The hybrids were isolated and subcultured until they either ceased division or achieved 100 population doublings (PD), our criterion for immortality. Detailed procedures for cell fusion, hybrid selection and isolation, cell culture, and determination of proliferative potential (PD accrued) have been described (10, 11, 13). In the case of G418 selection, the drug was used at a concentration of 1 mg/ml and the medium was changed twice weekly. Table 1 lists the cell lines studied, their origin, and source. All cell lines tested negative for the presence of mycoplasma by the Hoechst staining procedure (14). 143BTK$^-$ was additionally tested by Bionique Laboratories (Saranac Lake, NY) and found to be negative for mycoplasma. The strategy for assignment of a cell line to a complementation group is diagramed in Fig. 1. The cell lines GM639, GM847, and HT1080 had in our earlier study been assigned to the same group, designated A (11). Other cell lines were fused with one or more of these cell lines. The proliferative potential of hybrids was determined and the cell line was assigned to group A (hybrids proliferated without division cessation) or not group A (hybrids exhibited limited proliferation potential). The cell line HeLa assigned by this analysis to not group A was then selected to be representative of group B. The other cell lines assigned to not group A were fused with HeLa and, depending on the proliferative behavior of the hybrids, assigned to group B or not group B. 143BTK$^-$ served as the parent cell line representative of group C and A1698 served for group D. Assignment to a group was further confirmed by the fact that the cell lines within a group complemented representatives of the other groups. There-

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Abbreviations: PD, population doublings; XP, xeroderma pigmentosum; SV40, simian virus 40.

Table 1. Assignment to complementation groups for indefinite proliferation potential

Cell line	Description	Source	Group assigned
GM639	SV40-transformed skin fibroblasts	IMR	A
GM847	SV40-transformed skin fibroblasts (HPRT$^-$, Lesch Nyhan)	IMR	A
VA13	SV40-transformed lung fibroblasts	IMR	A
wtB	SV40-transformed keratinocytes	C. Noonan	A
A268IV	SV40-transformed amnion	J. Fogh	A
SVHF39	Origin-defective SV40-transformed bone fibroblasts	H. Ozer	A
GM2096SV9	Origin-defective SV40-transformed XP skin fibroblasts	D. Canaani	B
CW12 XP	SV40-transformed XP skin fibroblasts	R. Moses	A
CMV-Mj-HEL-1	Cytomegalovirus-transformed lung fibroblasts	F. Rapp	C
293	Adenovirus-transformed embryonic kidney	ATCC	Not A, not B, not C
W138-Ct1	Coirradiated lung fibroblasts	M. Namba	D
SUSM-I	4NQO-transformed liver fibroblasts	M. Namba	D
HT1080	Fibrosarcoma (N-*ras*$^+$)	R. Baker	A
143BTK$^-$	TE85 secondarily transformed by Kirsten mouse sarcoma virus (Ki-*ras*$^+$)	C. Croce	C
T98G	Glioblastoma	G. Stein	B
HeLa	Cervical carcinoma	ATCC	B
A549	Lung carcinoma	S. Aaronson	Not A, not B, not C
A2182	Lung carcinoma (Ki-*ras*$^+$)	S. Aaronson	D
EJ	Bladder carcinoma (H-*ras*$^+$)	R. Kucherlapati	A
J82	Bladder carcinoma	ATCC	B
A1698	Bladder carcinoma (Ki-*ras*$^+$)	S. Aaronson	D

IMR, Institute for Medical Research; ATCC, American Type Culture Collection; 4NQO, 4-nitroquinoline oxide.

fore, some cell lines were submitted to as many as four independent sets of fusions with cell lines representative of each group before group assignment was made. An important control in these studies was the fusion of double-marker cell line derivatives with the cell line of origin. As expected, all resulting hybrids proliferated indefinitely.

RESULTS

Fusions of various immortal cell lines with each other in some cases yielded hybrids that never ceased division (no complementation and assignment to the same group). In other fusions, however, we obtained hybrid clones that either had very limited doubling potential (<8 PD) or could proliferate more extensively (16–65 PD) and then ceased division. Since aneuploid parents were being fused, the possibility exists that some events other than true complementation resulted in the hybrids with low proliferative potential. We therefore restricted our analysis to hybrids that achieved 16 PD or more before loss of division cessation occurred. Division cessation was characterized by first a slowing in the rate of PD achieved and then followed by complete loss of ability to double the cell population. We have by this genetic analysis identified at least four complementation groups for indefinite division (Table 1). Details of the cell fusions done, number of hybrids analyzed, and the proliferative behavior of the hybrids are summarized in Tables 2–6.

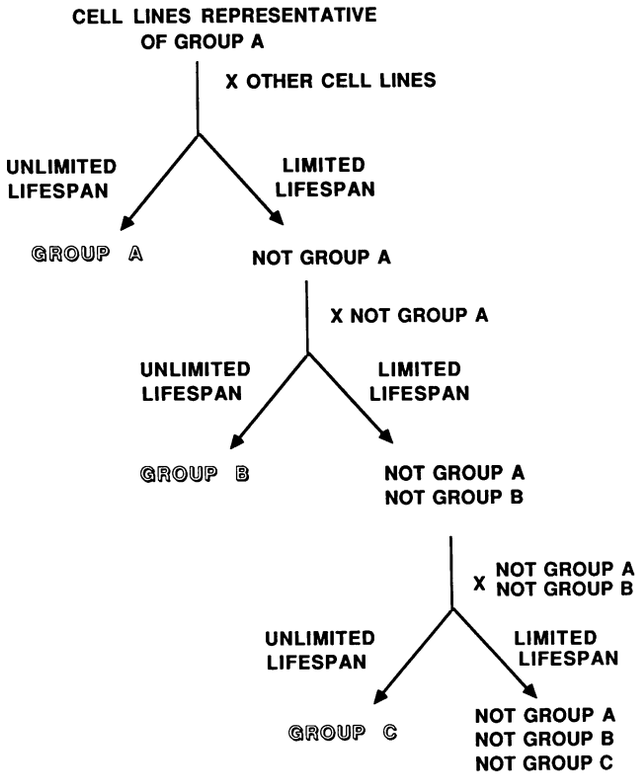

FIG. 1. Complementation group assignment strategy.

Assignment in some cases was made on the basis of four or more independent sets of fusions with cell lines representative of each group (see Table 6). For example, A1698, W138-CtI, and SUSM-I complemented lines in groups A–C, did not complement each other, and were assigned to the same group D. Complementation in this analysis was defined as loss of doubling potential in a hybrid population. This is a stringent definition as one would expect to occasionally obtain immortal hybrids from fusions that should complement but do not because of rapid chromosomal segregation with loss of critical growth regulatory genes. An example of this is the cell line GM2096SV9, which was assigned to "not group A" (Table 2) on the basis that 13 of 18 hybrids isolated from four independent fusions ceased division. The idea that the five immortal hybrids were the result of rapid segregation of chromosomes is supported by the observation that some of the nondividing hybrid populations maintained with weekly feedings for up to 2 months yielded immortal variants. Foci of dividing cells appeared in these populations at low frequencies (1 in 10^5 cells). In some cases, these cells divided to repopulate the culture, could achieve 100 PD, and had regained the immortal phenotype. In other instances, the cells would resume division but after a few PD again senesce, indicating that the changes in the cells that allowed for renewed cell division were not stable.

We included a variety of cell types in this study to determine whether parameters such as cell type, embryonal layer of origin, type of tumor, and oncogene expression would affect complementation group assignment. The vari-

Table 2. Fusion with cell lines representative of group A (GM639, GM847, HT1080)

Cell line	No. of hybrids	Range of PD at division cessation	No division cessation	Group assigned
GM639	12		All	A
GM847	12		All	A
VA13	12		All	A
wtB	10		All	A
A268IV	16		All	A
SVHF39	6		All	A
GM2096SV9	18	19–51	5/18	Not A
CW12 XP	5		All	A
CMV-Mj-HEL-1	10	16–22		Not A
293	10	16–20		Not A
W138-Ct1	5	20–65		Not A
SUSM-I	3	16–17		Not A
HT1080	4		All	A
143BTK$^-$	11	16–60		Not A
T98G	12	19–28		Not A
HeLa	11	16–65		Not A
A549	5	22–53		Not A
A2182	9	15–25		Not A
EJ	8		All	A
J82	4	18–22		Not A
A1698	7	20–22		Not A

ous immortal simian virus 40 (SV40)-transformed cell lines were selected to allow us to determine whether cell type (fibroblast versus epithelial) or embryonal layer of origin (mesodermal versus epidermal) would affect complementation group assignment. The results (Table 1) indicate that these parameters do not affect group assignment, since the immortal SV40-transformed cell lines derived from these different cells assign to group A.

Various cell lines known to contain DNA tumor viral genomes were analyzed to determine whether they would assign to the same group. The SV40 virus containing cell lines assign to group A, papilloma virus (HeLa) and cytomegalovirus (CMV-Mj-HEL-1) containing cell lines assign to group B, and an adenovirus (293) containing cell line assigns to group D. Therefore, different DNA tumor viruses mediate

Table 3. Fusions with cell lines representative of group B (HeLa)

Cell line	No. of hybrids	Range of PD at division cessation	No division cessation	Group assigned
GM639	11	16–65		Not B
GM847	4	17–24		Not B
wtB	12	18–24		Not B
A268IV	6	18–22		Not B
SVHF39	4	16–30		Not B
GM2096SV9	4		All	B
CW12 XP	5	21–25	2/5	Not B
CMV-Mj-HEL-1	6	18–47		Not B
293	2	31–52		Not B
W138-Ct1	5	20–58		Not B
SUSM-I	3	32–42		Not B
HT1080	5	18–27		Not B
143BTK$^-$	8	19–29		Not B
T98G	5		All	B
HeLa	5		All	B
A549	5	18–36		Not B
A2182	5	33–52		Not B
EJ	3	19–62		Not B
J82	6		All	B
A1698	5	18–56		Not B

Table 4. Fusions with cell lines representative of group C (143BTK$^-$)

Cell line	No. of hybrids	Range of PD at division cessation	No division cessation	Group assigned
GM639	8	19–29		Not C
CW12 XP	1	24		
CMV-Mj-HEL-1	6		All	C
293	6	20–52		Not C
W138-Ct1	4	29–39		Not C
SUSM-I	21	10–15		Not C
HT1080	5	18–27		Not C
HeLa	8	19–29		Not C
A549	7	10–12		Not C
A2182	64	10–15		Not C
J82	11	14–22		Not C
A1698	8	17–20	3/8	Not C

immortalization in human cells by different mechanisms. It is interesting that all of the immortal SV40-transformed cell lines we have studied, with the exception of GM2096SV9, assign to the same complementation group, indicating that this virus immortalizes different human cells by the same processes (Table 1; ref. 15). The exception, the cell line GM2096SV9, was derived from xeroderma pigmentosum (XP; group C) cells. Since it assigned to a group different from the other immortal SV40-transformed cell lines, we considered the possibility that XP, which involves DNA repair deficiencies, might have already generated some genetic changes in the normal cell that were exacerbated following introduction of SV40 DNA to yield the rare immortal variant that was isolated. To test this hypothesis, we included in our studies the immortal cell line CW12 XP, also derived from normal human XP (XP group C) fibroblasts following transfection with SV40 DNA. It assigned to group A along with the other immortal SV40-transformed cell lines. We then considered the fact that GM2096SV9 was obtained following the use of origin-defective SV40 virus. However, the cell line SVHF39, isolated following transfection with origin-defective SV40 viral DNA, assigned to group A. GM2096SV9 is unusual in that it is pseudodiploid and has very few genetic rearrangements unlike most immortal SV40-transformed cell lines. It is possible that this was why it assigned to a group other than the majority of the immortal SV40-transformed cell lines. As we proceed with further studies with these cell lines, the reason for the difference may become clear.

We chose to study various tumor-derived cell lines to determine whether, for example, all carcinomas would assign to one group that was distinct from sarcomas or whether all bladder carcinomas would assign together and different from lung carcinomas. The results demonstrate that type of tumor does not affect group assignment.

Finally, since oncogenes have been implicated as immortalizing genes for rodent cells, we also included human cell lines expressing activated oncogenes. A role for activated oncogenes in the immortalization process of human cells is

Table 5. Fusions with cell lines representative of group D (A1698)

Cell line	No. of hybrids	Range of PD at division cessation	No division cessation	Group assigned
GM639	7	20–22		Not D
HeLa	5	18–56		Not D
143BTK$^-$	8	17–20	3/8	Not D
SUSM-I	3		All	D
W138-Ct1	6		All	D
A2182	5		All	D

Table 6. Summary of results of all fusions

	A								B					C		D					
	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p	q	r	s	t	u
a	−	−	−	−	−	−			−	+		+		+	+	+	+	+	+		+
b	−	−	−			−	−	−		+	+		+	+	+					+	
c	−	−							−	+											
d	−																				
e	−									+											
f	−	−								+											
g		−								+											
h		−								+											
i	−		−							+											
j	+	+	+		+	+	+	+	+	−	−	−	−	+	+	+	+	+	+	+	+
k		+								−											
l	+									−											
m		+								−											
n	+	+	+							+					−	+	+	+	+	+	+
o	+	+								+				−							
p	+									+				+			−	−	−		
q	+									+				+		−					
r	+									+				+		−					
s	+		+							+				+		−					
t		+	+							+				+							
u	+									+				+							

a, GM639; b, GM847; c, HT1080; d, VA13; e, wtB; f, A268IV; g, SVHF39; h, CW12 XP; i, EJ; j, HeLa; k, T98G; l, J82; m, GM2096SV9; n, 143BTK$^-$; o, CMV-Mj-HEL-1; p, A1698; q, W138-Ct1; r, SUSM-I; s, A2182; t, A549; u, 293.

not immediately evident from the results we have obtained, since there is no correlation between oncogene expression and group assignment. It is, however, interesting that cell lines containing an activated N-*ras* or H-*ras* oncogene assign to the same group as the SV40 T-antigen-expressing cell lines.

DISCUSSION

The first and most exciting result of these data is that we have assigned a large number of immortal human cell lines to a few complementation groups for indefinite division. This indicates that immortalization does not result from random events but rather from changes in specific genes. It could be argued that what we are observing is not true complementation but rather loss of some essential immortalizing gene. This could possibly explain the hybrid clones with very limited division potential (<8 PD) that we obtained but did not include in the analysis. However, it is difficult to explain the sudden loss of division potential in a population of 10^5–10^6 cells, which has achieved as many as 65 PD, on the basis that some gene critical to immortalization was lost in all of the cells at the same time. The cells were not in contact at the time of loss of doubling potential, eliminating the possibility that surrounding cells affected a possible immortal cell, as has been reported in other systems (16, 17). The most obvious interpretation is that the normal growth regulatory gene complement is now restored, resulting in cellular senescence.

Other laboratories studying immortal–immortal human hybrids have also reported hybrids having limited division (18–20). However, the emphasis of the studies was on the tumorigenic behavior of the hybrids and minimum attention was paid to limited division hybrids. In one study in which a clone derived from HeLa cells (D98) was fused with various human tumors, the authors predicted that complementation groups for immortality would not be tumor type specific on the basis of their observations of hybrid colonies that stopped dividing (17). This is what we have found. The lack of correlation between complementation group assignment and cell type, embryonal layer of origin, and type of tumor indicates that a few specific genes or sets of genes are involved in normal growth control and modified to result in immortalization. This conclusion is additionally supported by the fact that we have not been able to make a dual assignment of any of the cell lines we have studied, despite their being in culture for many years.

In a separate series of experiments, we and others have demonstrated that senescent human cells express a surface membrane-associated protein inhibitor of initiation of DNA synthesis (21, 22) and contain a high abundance of DNA synthesis inhibitory messenger RNAs that are not present in young cells (23). We hypothesize that as human cells age in culture, they begin to produce a protein that blocks their entry into the S phase of the cell cycle. They respond to this inhibitor and are therefore unable to proliferate, even in the presence of excess mitogenic factors. On the basis of this hypothesis, we can propose two ways by which cells might become immortal. (*i*) They might lose the ability to produce an effective inhibitor of DNA synthesis. This might be the result of a point mutation in the gene coding for the inhibitor, loss of the genetic information coding for the inhibitor (deletion, translocation, or chromosome loss), defects in the cis- or trans-acting factors controlling the expression of the inhibitor, or loss of necessary posttranslational modifications. (*ii*) They might lose the ability to respond to the inhibitor. Until we know more about the mode of action of the inhibitor protein we can only speculate on how this might occur. For example, a receptor-like molecule might be necessary for recognition of the inhibitor activity, and loss of this receptor would result in cellular immortality.

If this protein inhibitor is indeed involved in cellular senescence and changes in expression result in immortalization, it would be a prime candidate for a tumor suppression gene. Existence of normal cellular genes that suppress tumorigenicity is now accepted and Sager and coworkers (24) have demonstrated that cellular senescence is one of the mechanisms by which tumor suppression occurs. Identification of the genes modified to result in immortalization should therefore increase our understanding of cellular aging and tumorigenicity. Assignment of immortal cell lines to specific groups now allows us to proceed with a focused approach to do just that. By studying cell lines within each group we can

determine what common changes have occurred in these cells to result in immortalization. By using antibody and cDNA probes against the inhibitor of DNA synthesis, we can determine whether changes such as those described above have occurred to result in immortality. We can begin cytogenetic studies to identify chromosomal aberrations common to the cell lines within a group and take approaches that have proven successful in identifying chromosomes carrying tumor suppression genes, such as microcell fusion, to determine what genes have been modified in the immortal cell. Such studies have not been possible before because of lack of grouping of immortal cells.

The fact that all but one of the immortal SV40-transformed cell lines we have studied assign to the same group provides another avenue to identify the normal cell regulatory genes. Data from the studies here and earlier work from our and other laboratories indicate that the expression of SV40 T antigen is not sufficient to maintain immortalization in human cells, since cells that are expressing functional T antigen exhibit limited proliferation potential (25–34). The question then is whether any viral genes are involved in maintenance of immortalization of SV40-transformed human cells or whether events such as aneuploidy, which occur subsequent to the entry of the virus into the cells and are independent of any viral genes or products, are responsible for maintenance of immortalization. If T antigen is needed to maintain the immortal phenotype, one set of candidate cellular genes with which T antigen could cooperate to maintain the immortal phenotype would be the "*ras*-like" family of oncogenes. We raise this possibility because cell lines expressing the N-*ras* or H-*ras* oncogenes assign to the same group as the immortal SV40-transformed cell lines we have studied.

In conclusion, these data support the hypothesis that cellular senescence is the result of a genetic program and that recessive changes in the genes, sets of genes or processes involved, yield immortal cells. The identification of a limited number of complementation groups for indefinite division indicates that it will be feasible to identify the genes. The fact that there is no correlation between cell type, embryonal layer of origin, and type of tumor and that we have not yet assigned any cell line to more than one group indicates that we are dealing with highly specific and small number of genes or processes. These results also account for the fact that human cells are very stable *in vitro* and that the frequency of immortalization in these cells is very low.

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