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Transfer of Anchorage Independence by Isolated Metaphase Chromosomes in Hamster Cells

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Summary

The cellular property of being able to grow on agar (*aga*$^+$) or to show anchorage independence has been transferred by means of metaphase chromosomes from CHO cells to BHK and other permanent transformed hamster lines unable to grow on agar. As with other genetic markers, the transferents are unstable when grown under non-selective conditions. The *aga*$^+$ transferents are tumorigenic, providing further evidence for the association between the ability to grow in agar or anchorage independence and tumorigenicity. Evidence has been obtained in these experiments for the existence of at least two discrete events in the transformation of normal into tumorigenic cells. The ability to transfer and select for the *aga*$^+$ marker in recipient cells indicates that tumorigenicity behaves dominantly phenotypically.

Introduction

Although several alterations in phenotype have been associated with the transformation of normal into cancer cells (Ossowski et al., 1973; Rowley, 1974; Hynes, 1976; Tucker et al., 1977), present evidence indicates that the ability to produce tumors in animals is best correlated with anchorage independence (MacPherson and Montagnier, 1964; Di Mayorca et al., 1973; Kakunaga, 1973; Freedman and Shin, 1974; Shin et al., 1975; Bouck and Di Mayorca, 1976; Jones et al., 1976). For example, Shin et al. (1975), using a number of normal and transformed mouse and rat cells, showed that growth properties such as optimum cell density, and serum requirements, as well as the presence or absence of viral T antigen, were not correlated with the ability to produce tumors in nude mice, whereas the ability to show anchorage independence was. Jones et al. (1976) found a similar correlation between the capacity of mouse cells to form colonies in soft agar and tumorigenicity of the cells in neonatal animals.

The recently developed technology in which metaphase chromosomes are used to transfer specific genetic markers to appropriate recipient cells (McBride and Ozer, 1973; Burch and McBride, 1975; Willecke and Ruddle, 1975; Wullems, van der Horst and Boostma, 1975; Degnen et al., 1976; Willecke et al., 1976; Spandidos and Siminovitch, 1977a, 1977b, 1977c) provides an opportunity to examine the genetic basis of anchorage independence in greater detail. There is evidence that only a small chromosomal fragment of <0.2% of the total genome is transferred in such experiments (McBride and Athwal, 1976; Willecke et al., 1976). Thus if the property of anchorage independence could be transferred by metaphase chromosomes, then it should be possible to delineate more clearly the associated cellular phenotypes.

In previous studies we have developed techniques whereby dominant markers such as those governing methotrexate (*mtx*^{R}) and ouabain (*oua*^{R}) resistance, (Spandidos and Siminovitch, 1977a), and prototrophic markers such as *glyA*$^+$, *glyB*$^+$ and *gat*$^+$ (Spandidos and Siminovitch, 1977b), can be transferred by CHO metaphase chromosomes into recipient CHO cells with relatively high efficiency (Spandidos and Siminovitch, 1977a, 1977b, 1977c). In this paper we show that the property of anchorage independence (*aga*$^+$) can be similarly transferred. [We have defined anchorage independence as the ability to grow (produce colonies) on agar; hence the term *aga*. In our experience, such cells are able to grow in suspension and are therefore anchorage-independent.] Several different types of recipient cells have been used, and the resulting transferents behave similarly to those found when biochemically dominant markers are used.

Results

Transfer of Anchorage Independence (*aga*$^+$) from CHO to BHK Cells

CHO cells grow well in suspension and show plating efficiencies (PE) on agar similar to those found on plastic plates. Our line of BHK cells, BHK21, gives very low PEs on agar (2×10^{-6}). This combination of properties provided the opportunity to examine whether the ability to grow on agar (*aga*$^+$), or anchorage independence, could be transferred from cell to cell by means of metaphase chromosomes. We incubated purified metaphase chromosomes from CHO cells with BHK21 cells, which we then plated on agar as described in Experimental Procedures. The results from three independent experiments are shown in Table 1. Colonies appeared at a frequency of approximately 2×10^{-5} per recipient cell (lines 1-2). We saw no colonies on the agar plates in the absence of recipient cells (lines 5 and 6). We obtained a small number of colonies, however, when no metaphase chromosomes were added to the recipient cells, indicating that there is a small spontaneous frequency of conversion to *aga*$^+$ in BHK21 cells (lines 3 and 4). This frequency was much lower than that found in

the experimental situation (lines 1 and 2), indicating that most of the latter colonies were indeed transferents, and that the property of anchorage independence had been transferred to BHK cells by means of the CHO metaphase chromosome.

Karyotypic analysis of ten transferent clones showed a modal chromosome number of forty four chromosomes, which is characteristic of BHK, but not CHO, cells.

Transfer of the *aga*$^+$ Property to Other Chinese Hamster Cell Lines Using CHO Metaphase Chromosomes

After we obtained evidence that the *aga*$^+$ markers could be transferred to BHK21 cells by means of metaphase chromosomes, we next tested the generality of the observation by using two other recipient lines, both derived from Chinese hamster primary cells. The origins of these lines, CHLTF1 and CHLRFStran4, are described in Experimental Procedures. Briefly, CHLTF1 is a hamster line which has undergone spontaneous transformation in vitro, and CHLRFStran4 is a hamster line which has been rescued from senescence by chromosome transfer, and which has probably undergone an early step in transformation (D. A. Spandidos and L. Siminovitch, manuscript in preparation). As shown in Table 2, we again obtained colonies when CHO metaphase chromosomes were added to CHLRFStran4 cells, and produced selection on 1% noble agar (line 1). A number of controls were included in this experiment. We obtained no colonies when metaphase chromosomes from primary Chinese hamster cells, CHOP6, were used as donors (line 2), or when we added CHO metaphase chromosomes to CHOP6 cells (line 3). We added no chromosomes at all to CHLRFStran4 cells (line 4).

We also obtained transferent colonies when we used CHLTF1 cells as recipients (data not shown). This positive result will be illustrated by the data to be described in which we used fractionated chromosomes.

Analysis of the karyotypes of a number of these transferent clones indicated that their banding pattern was identical to normal diploid hamster cells.

Studies on the Stability of the Transferred Genetic Material

Our previous results (Spandidos and Siminovitch, 1977a, 1977b, 1977c), as well as those obtained from other laboratories (McBride and Ozer, 1973; Willecke and Ruddle, 1975; Wullems, Van der Horst and Boostma, 1975; Degnen et al., 1976), have shown that most transferents are unstable and that the markers are lost on growth in nonselective medium. It was next of interest to examine the behavior of the *aga*$^+$ phenotype in this respect. To study the stability of the *aga*$^+$ marker, we subcloned the transferent clone, BHKaga$^+$tran101, and the spontaneous *aga*$^+$ mutant, BHKaga$^+$201, on agar plates, and we grew three subclones (A, B and C) of each on plastic plates for 30 days. At intervals, we trypsinized the cells and examined their ratios of plating efficiencies (agar plate/plastic plate). The results are shown in Figure 1. All three subclones of the BHKaga$^+$201 clone showed considerable stability, while only the BHKaga$^+$tran101A subclone of the transferent clone was stable. Subclones BHKaga$^+$tran101B and BHKaga$^+$tran101C showed a relative plating efficiency (agar plate/plastic

Table 1. Transfer by Purified Metaphase Chromosomes of CHO Marker(s) That Determine the Ability of Cells to Grow on Agar Plates (*aga*$^+$) into BHK21 Cells

Donor	Recipient	Agar Plate Selection	Colonies per 1 X 10^6 Cells Experiment Number 1	2	3
(1)[a] CHO	BHK21	0.5% agar	15	21	18
(2) CHO	BHK21	1.0% agar	13	19	25
(3)[b]	BHK21	0.5% agar	0	2	3
(4)	BHK21	1.0% agar	1	2	0
(5)[c] CHO		0.5% agar	0	0	0
(6) CHO		1.0% agar	0	0	0

[a] For (1) and (2), a total number of 5 X 10^6 cell equivalent of chromosomes were added to 2 X 10^6 cells. After adsorption and growth for 2 days at 37°C, 1 X 10^6 cells were plated on agar (Kuroki, 1975). The plates consisted of Noble agar at the concentrations indicated, in α complete medium (Stanners, Glicieri and Green, 1971) and 10% FCS. Cells were plated at a concentration of 1 X 10^5 cells per 100 mm agar plate.

[b] For (3) and (4), a total number of 1 X 10^6 cells were plated (1 X 10^5 cells per plate).

[c] For (5) and (6), 1 X 10^6 cell equivalent chromosomes were plated directly (1 X 10^5 cell equivalent chromosomes per plate).

Table 2. Transfer of the *aga*$^+$ Marker into CHLRFStran4 Cells Using Purified Metaphase Chromosomes of CHO Cells

Donor for Chromosome Isolation	Recipient Cells	Colonies per 1 X 10^6 Cells Experiment Number 1	2	3
(1)[a] CHO	CHLRFStran4	8	3	4
(2) CHOP6	CHLRFStran4	0	0	0
(3) CHO	CHLP6	0	0	0
(4)[b]	CHLRFStran4	0	0	0
(5)[c] CHO		0	0	0

Conditions for the experiments shown in [a](rows 1-3), [b](4) and [c](5) were the same as described in Table 1, except that the selection medium used was α special medium (McBurney and Whitmore, 1974) containing 1% Noble agar and 15% DFCS.

plate) of <1% after 30 days of growth in liquid medium. This type of result was obtained for several different *aga*$^+$ clones as summarized in Table 3. We grew a number of gene transferent clones, as well as spontaneous *aga*$^+$ mutants, for 30 days on plates in liquid medium (nonselective conditions), and determined the ratios of plating efficiencies (agar plate/plastic plate) at that time. As may be seen in Table 3, while the *aga*$^+$ marker was stable in spontaneous mutants of BHK21 cells, it was unstable in gene transferent cells.

Location of the *aga*$^+$ Marker

In earlier work, we showed that metaphase chromosomes from CHO cells could be fractionated into three size classes, called A, B and C, by sucrose gradient centrifugation, and that the *mtx*^{RIII} marker could be located in the medium size B class of chromosomes, and the *oua*^{R} marker in the large size A class (Spandidos and Siminovitch, 1977a). To determine whether the *aga*$^+$ marker could be similarly located in a particular size class, we fractionated CHO metaphase chromosomes as described earlier, and tested the ability of each fraction to transfer the *aga*$^+$ markers to BHK21 and CHLTF1 cells. The results may be seen in Table 4. The major *aga*$^+$ activity was found in the large size A class of chromosomes (lines 1 and 4). For BHK

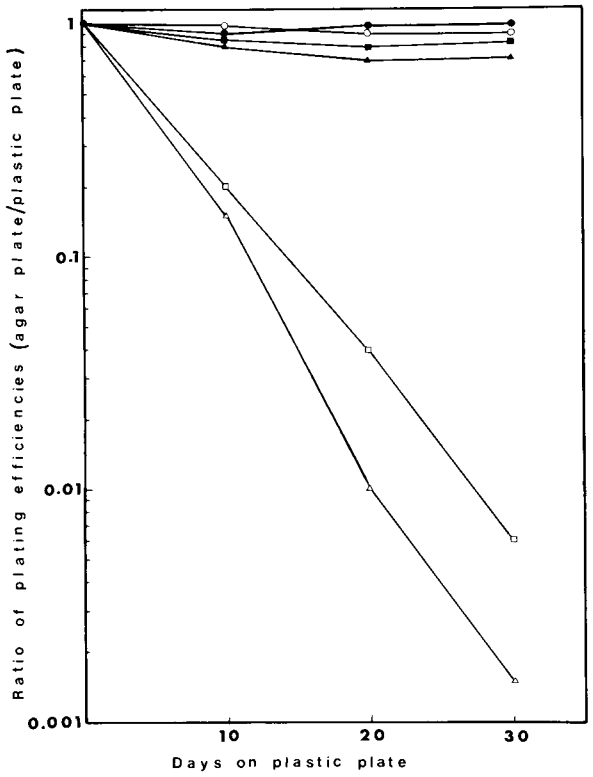

Figure 1. Stability of the aga$^+$ Marker in BHK Mutant and Transferent Cells

The graph depicts the stability of the *aga*$^+$ marker under nonselective conditions in three subclones (A, B and C) of the BHKaga$^+$ tran101, and in three subclones (A, B and C) of the mutant BHKaga$^+$201. At time 0, all cell lines that had been maintained under selective conditions (1.0% agar plate) were transferred to nonselective conditions (plastic plate). Cells were removed at the indicated times and tested for plating efficiencies in selective and nonselective conditions. The relative plating efficiencies at time 0 were not tested but were assumed to be 1. BHKaga$^+$tran101A (○), 101B (□), 101C (△), BHKaga$^+$201A (●), 201B (■) and 201C (▲).

Table 3. Comparison of Stabilities of the *aga*$^+$ Marker in Spontaneous *aga*$^+$ Mutants and in *aga*$^+$ Transferents after Growth for 30 Days on Plastic Plate

Cell Line	Ratio of Plating Efficiencies Agar Plate[a] per Plastic Plate[b]
CHO	8×10^{-1}
BHK23	1×10^{-6}
BHKaga$^+$201[c]	8×10^{-1}
BHKaga$^+$202	1×10^{0}
BHKaga$^+$203	7×10^{-1}
BHKaga$^+$204	5×10^{-1}
BHKaga$^+$205	9×10^{-1}
BHKaga$^+$tran101	3×10^{-2}
BHKaga$^+$tran102	1×10^{-1}
BHKaga$^+$tran103	4×10^{-3}
BHKaga$^+$tran104	8×10^{-4}
Bhkaga$^+$tran105	1×10^{-2}
BHKaga$^+$tran106	2×10^{-2}
BHKaga$^+$tran107	6×10^{-3}

[a] α complete medium containing 1% Noble agar and 10% FCS.
[b] α complete medium containing 10% FCS.
[c] BHKaga$^+$201–205 are *aga*$^+$ cells which arose spontaneously, and BHKaga$^+$tran101–107 are transferents.

Table 4. Transfer of the *aga*$^+$ Marker into BHK21 or CHLTF1 Cells Using Fractionated Metaphase Chromosomes of CHO Cells

Chromosomal Fraction	Recipient Cells	Colonies per 1 X 10^6 Cells Experiment Number 1	2	3
(1)[a] A	BHK21	34	29	38
(2) B	BHK21	2	0	3
(3) C	BHK21	0	1	2
(4) A	CHLTF1	7	11	
(5) B	CHLTF1	0	0	
(6) C	CHLTF1	0	0	
(7)[b]	BHK21	1	0	2
(8)	CHLTF1	0	0	
(9)[c] A+B+C		0	0	0

Conditions for the experiments shown in [a](rows 1-6), [b](7, 8) and [c](9) were the same as described in the legend to Table 1.

cells, the number of transferents found using the B and C size chromosomes fractions (lines 2 and 3) was similar to that found in the control where we used no chromosomes (line 7). Since the data shown in lines 2 and 3 provide another control for the number of spontaneous *aga*$^+$ colonies found in BHK21 cultures, these results provide a further indication that the colonies observed in the experimental situation represent transferents. The results with CHLTF1 are more clean-cut, since no colonies at all were observed in the B and C size fractions of the chromosome preparations (lines 5 and 6).

Phenotypic Properties of *aga*$^+$ Transferent Cells

After we had shown that the properties of anchorage independence could be transferred by metaphase chromosomes, we were next interested in examining some of the phenotypic properties of such clones.

One property that has been associated with anchorage independence is agglutinability with concanavalin A (Inbar and Sachs, 1969). As may be seen in Figure 2, CHO cells are highly agglutinable by ConA, whereas BHK21 cells show very little such activity. BHKaga$^+$ cells, formed either by spontaneous mutation or by transfer of metaphase chromosomes from CHO cells, were highly agglutinable, and thus the *aga*$^+$ property seems to carry this phenotype with it.

As indicated in the Introduction, a more important association that has been observed in the past is that between the *aga*$^+$ property and tumorigenicity. To test for this association, we injected newborn Syrian hamsters with 10^3 and 10^7 cells from BHK21, BHKaga$^+$tran and BHKaga$^+$ spontaneous mutant cultures. We observed palpable tumors in animals injected with the *aga*$^+$ cells 30 days after inoculation with 10^3 cells. We found no tumors in the animals injected with 10^3 BHK21 cells. We obtained tumors, however, when 10^7 such cells were injected, presumably because of the presence of *aga*$^+$ cells in these populations (Jarrett and McPherson, 1968; DiMayorca et al., 1973). When we injected 10^7 of the *aga*$^+$ cell populations, we obtained tumors at 8–10 days. We conducted similar experiments with the Chinese hamster cell lines, except that we used Chinese hamsters as the test animals. As seen in Table 5, CHO cells and the two *aga*$^+$ transferent clones were highly tumorigenic, whereas the primary CHLP6 strain and the CHLTF1 cell lines, as well as the line "rescued" from senescence CHLRFStran4, all of which are *aga*$^-$, were inactive. Thus these results support the contention that tumorigenicity is associated with the property of anchorage independence. Table 5 also provides data on the saturation density observed in the various Chinese hamster lines. We found no obvious correlation between saturation density and anchorage independence or tumorigenicity, a result previously found by others (Shin et al., 1975).

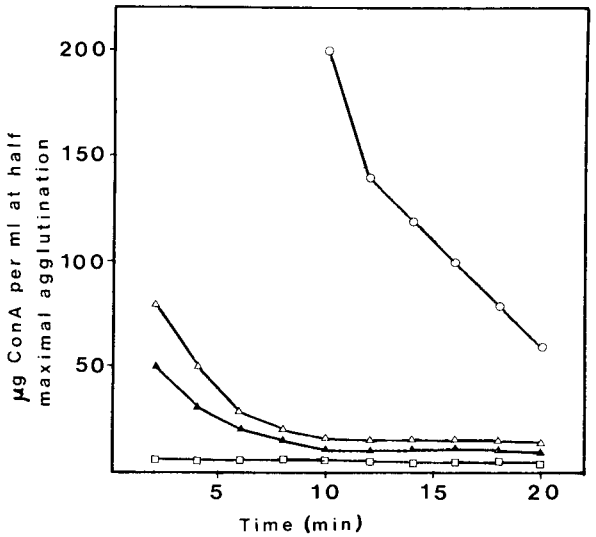

Figure 2. Agglutinability with ConA of Transferent and Other Hamster Cell Lines

The graph depicts agglutination of BHK, BHKaga$^+$tran101, BHKaga$^+$201 and CHO cells as a function of time. (○——○) BHK; (▲——▲) BHKaga$^+$tran101A; (△——△) BHKaga$^+$201A; and (□——□) CHO cells.

Discussion

In previous work involving chromosome transfer, evidence for such transfer has included the demonstration of the presence of the specific donor cell gene product in the recipient cells, and the instability of the marker in transferent cells as compared with new mutations (McBride and Ozer, 1973; Willecke and Ruddle, 1975; Wullems et al., 1975; Degnan et al., 1976; Spandidos and Siminovitch, 1977a, 1977b). Information of the former kind is difficult to obtain for the *aga*$^+$ marker. The evidence that the new *aga*$^+$ isolates represent transferents, however, is extremely strong: first, for two of the recipient lines, we have never observed *aga*$^+$ colonies in the absence of donor chromosomes; second, the *aga*$^+$ transferents are unstable whereas mutations of *aga*$^+$ are stable; and third, we have shown that the *aga*$^+$ marker can be localized to a specific chromosomal size class. In addition to providing evidence for specificity and further controls for the lack of *aga*$^+$ colony formation in the absence of active chromosomes, this result also tends to rule out any hypothesis which proposes that chromosomes per se may have increased the mutation rate of *aga*$^-$ to *aga*$^+$ in the recipient cells.

The results described in this paper therefore

indicate strongly that the property of being able to form colonies on agar (*aga*$^+$), or anchorage independence, can be transferred to recipient cells by means of metaphase chromosomes. Although there were several previous indications that this cellular characteristic behaved as a genetic entity (McPherson and Montanier, 1964; Di Mayorca et al., 1973; Miyashita and Kakunaga, 1975; Bouck and Di Mayorca, 1976), our data provide further strong evidence for this view. This evidence includes the fact that frequency of transfer of *aga*$^+$ to recipient cells is in the same range as that for other unique genetic markers, the ability to localize the *aga*$^+$ marker to a specific chromosomal size class, and the fact that the *aga*$^+$ transferents show the same type of instability found previously in our lab for genetic loci such as *mtx*^{R} and *oua*^{R} (Spandidos and Siminovitch, 1977a, 1977b, 1977c), and by others for other loci (McBride and Ozer, 1973; Willecke and Ruddle, 1975; Wullems, Van Der Horst and Boostma, 1975; Degnen et al., 1976).

Our data also provide direct support for the view that tumorigenicity of cell cultures is associated with the anchorage-independent phenotype. Since present evidence indicates that only a small fraction of the genome (~0.2%) is transferred in experiments involving chromosome transfer, (McBride and Athwal, 1976; Willecke et al., 1976), it seems probable that both copies are carried by the same gene. There is, of course, a possibility that the *aga*$^+$ and tumorigenicity markers are separate but linked genes; there is no a priori reason, however, for this assumption.

The data of Tables 2 and 5 are of special interest in respect to the processes of evolution of *aga*$^+$ cells and of tumorigenicity. The CHLRFStran4 cell line consists of cells which have been "rescued" from senescence by the addition of metaphase chromosomes from CHO cells, followed by selection for colony formation. As will be described elsewhere, such cells have a slightly altered morphology and appear to be transformed (D. A. Spandidos and L. Siminovitch, manuscript in preparation). The CHLTF1 cell line became established simply by culture in the laboratory, and is also transformed morphologically. Both of these lines can be converted to the *aga*$^+$ phenotype by CHO chromosome transfer. In addition, we have found that cells transformed in vitro by benzopyrene, and which have not progressed to the point where they can grow on agar, can also be converted to *aga*$^+$ cells by metaphase chromosome transfer (D. A. Spandidos and L. Siminovitch, unpublished observations). In all these systems, when similar experiments were carried out using primary hamster cells as recipients (line 3, Table 2), no transferents were obtained. These results indicate, therefore, that at least two discrete events (or genes) are involved in the formation of *aga*$^+$ cells. The observation that transformation to anchorage independence may occur by a series of discrete steps has been made previously (Dulbecco, 1976). The same two events seem to be involved in the evolution of tumorigenicity, since the CHLRFStran4 and CHLTF1 cells are not tumorigenic, whereas the CHLRFStran4aga$^+$tran6 and CHLTF1aga$^+$tran1 cells are. These data again underline the association between the aga$^+$ marker and tumorigenicity.

CHO cells contain C-type particles which can be visualized by electron microscopy, and can be tested for reverse transcriptase activity (Lieber et al., 1973). It could be argued, therefore, that the conversion of BHK21 cells, or the other strains, to anchorage independence was due to transfer of such C-type particles. This was improbable because the centrifugation procedures used to prepare metaphase chromosomes, and particularly the fractionated samples (for example, Table 4), should have eliminated any such particles. In addition, we have found that whereas CHO cells show considerable transcriptase activity, two other *aga*$^+$ populations, BHKaga$^+$201A and BHKaga$^+$tran101A

Table 5. Properties of Chinese Hamster Cell Lines

Cell Line	Saturation Density[a] Cells per cm^2 X 10^4	Ability to Grow on Agar[b]	Tumorigenicity[c]
CHO	22	8×10^{-1}	6/6
CHLP6	12	$<1 \times 10^{-7}$	0/5
CHLRFStran4	31	$<1 \times 10^{-7}$	0/6
CHLRFStran4aga$^+$tran6	54	5×10^{-1}	5/6
CHLTF1	17	$<1 \times 10^{-7}$	0/5
CHLTF1aga$^+$tran1	25	6×10^{-1}	5/5

[a] All cells were grown in α-medium containing 10% FCS at 37°C.
[b] All cells were tested for growth on agar plates containing 1% Noble agar and 10% FCS in α-medium.
[c] The ratio of animals with tumors per animals tested 2 months after subcutaneous injection of 3 day old newborn Chinese hamsters with 1×10^6 cells.

Table 6. Yields of Chromosomes

Step	% Cells or Cell Equivalent of Chromosomes Recovered[a]
(1) Plated Synchronized Cells	100
(2) Detached Mitotically Arrested Cells	70
(3) Chromosome Precipitate (1200 X g)	55
(4) First Sucrose Gradient	45
(5) Second Sucrose Gradient	40

[a] Determined by using a hematocytometer and phase microscope.

(see Table 3), show activities similar to that seen in BHK21 cells. Thus it is highly improbable that the transfer of the *aga*$^+$ markers is due to "mass infection" with C-type viruses. The transfer of a C-type genome is not ruled out, however, nor is the possible contamination of the chromosome preparations with membrane-associated viruses.

The ability to transfer the *aga*$^+$ marker has both theoretical and practical implications. There are several cell lines available which provide particularly suitable experimental material for specific biological experiments, but which do not grow in suspension. It seems possible that these cells have already undergone the primary event alluded to earlier, which would make it feasible to convert them to *aga*$^+$ by chromosome transfer, and thus to produce cultures which would grow in suspension. This would have extensive practical utility.

Our results are of interest regarding whether malignancy behaves as a dominant or a recessive trait. The fact that the *aga*$^+$ genome is expressed and can be selected in recipient cells provides very strong evidence that this property, and the associated tumorigenicity, behave phenotypically in a dominant fashion. There has been considerable controversy about this question based on studies on whether malignancy is, or is not, suppressed in somatic cell hybrids (Ozer and Jha, 1977; Minna, 1977). Ozer and Jha have indicated some of the difficulties inherent in the interpretation of experiments involving cell hybrids. These equivocations do not apply to work in the chromosome transfer system, however, and it seems clear that when one examines the dominance or recessive question in this way, the most credible conclusion which can be drawn is that malignancy acts dominantly.

The ability to delineate the two presumed stages leading to tumorigenicity by discrete chromosomal transfer steps should provide excellent experimental material to examine the phenotypes associated with each stage, and to examine in greater detail the genetic basis for the process of tumorigenesis in vitro.

Experimental Procedures

Cell Lines and Culture Conditions

We used a number of recipient cell lines or strains for our transfer experiments. A Syrian hamster fibroblast cell line, BHK21/13 (Stoker and MacPherson, 1964), was obtained from Dr. H. Murialdo who obtained it originally from Dr. G. Di Mayorca; this line is hereafter called BHK21. Chinese hamster ovary, CHOP6, and Chinese hamster lung, CHLP6, cell strains were derived from explants of adult female Chinese hamsters, and were provided by Dr. D. Hoar and Miss C. Campbell. They were both subcultured by 1:3 dilution and used at their sixth transfer.

CHLTF1 is a Chinese hamster lung cell line obtained by C. Campbell from primary Chinese hamster lung cells as a result of a spontaneous transformation. CHLRFStran4 is a cell line derived by "rescue" of senescent Chinese hamster lung cells by transfer of metaphase chromosomes from the CHO cell line (D. A. Spandidos and L. Siminovitch, manuscript in preparation). Both CHLTF1 and CHLRFStran4 cell lines do not grow in suspension, and no colonies were observed on agar plates for each cell line when >5 X 10^7 cells were plated. The donor cell line for most experiments was *Pro*$^-$CHO, a proline-requiring Chinese hamster ovary (CHO) auxotroph hereafter called CHO. It has been cultured in our laboratory for a number of years (Thompson and Baker, 1973). In conformity with the nomenclature described earlier, clones containing transferred metaphase chromosome material (transferents), which also showed anchorage independence—that is, the ability to grow on agar (*aga*$^+$)—are indicated as *aga*$^+$ tran101, *aga*$^+$tran102 and so forth (Spandidos and Siminovitch, 1977a).

The cell culture techniques have been described in detail elsewhere (Thompson and Baker, 1973). The agar plate culture technique for mammalian cells has also been described in detail (Kuroki, 1975).

The isolation, transfer and fractionation of chromosomes have been outlined briefly (Spandidos and Siminovitch, 1977a) and are described in greater detail here.

Isolation of Chromosomes

Logarithmically growing CHO cells in suspension were diluted with fresh medium before they had reached 2 X 10^5 cells per ml. The suspension cultures were synchronized by placing them at 34°C for 22 hr in the presence of 2 mM thymidine (thymidine block) (Boostma, Burke and Vos, 1964). The cells were then centrifuged and resuspended in calcium-free medium containing 0.06 μg/ml colcemid, plated in 75 cm^2 area plastic bottles (5 X 10^6 cells per bottle) and incubated at 34°C for another 12 hr. The bottles were then shaken manually to detach mitotic cells, which were released in the medium and collected by centrifugation. The yield of mitotic cells was 50–70% of the total cell population under these conditions. These cells were resuspended in hypotonic buffer (75 mM KCl) at 4°C for 15 min at a concentration of 5 X 10^6 cells per ml, centrifuged at 125 X g, washed with chromosome isolation buffer (pH 6.5) containing 0.5 mM $CaCl_2$, 0.1 mM PIPES and 1.0 M hexyline glycol (Wray and Stubblefield, 1970), and resuspended in chromosome isolation buffer containing 0.5% NP40 detergent at a concentration of 5 X 10^6 cells/ml. The cell suspension was then passed five times through a 22-gauge needle to break the cell membranes. The chromosomes were collected by centrifugation at 1200 X g and resuspended in 5 ml of chromosome isolation buffer by gentle pipetting.

Chromosomes were further purified by velocity sedimentation in sucrose gradients. 5 ml of the chromosome suspension were layered over a 5–40% sucrose gradient in chromosome isolation buffer (7 ml), which in turn was layered over a 3 ml cushion of 40% sucrose in chromosome isolation buffer in 15 ml plastic conical tubes. Centrifugation was performed at 125 X g for 30 min at 20°C in an MSE centrifuge. Under these conditions, contaminating nuclei were pelleted and the chromosomes formed a rather sharp peak near the surface of the 40% sucrose cushion.

All fractions containing chromosomes were pooled together, diluted up to 15 ml with chromosome isolation buffer and centrifuged at 1200 X g for 20 min. Such preparations were virtually devoid of isolated nuclei and whole cells when examined with a phase microscope. A separation of chromosomes into three different size classes (A = large, B = medium and C = small) was achieved by further purification on a 10–40% sucrose gradient after centrifugation at 50 X g for 40 min as described previously (Mendelsohn, Moore and Salzman, 1968; Spandidos and Siminovitch, 1977a). A summary of the separation and yields of chromosomes is shown in Table 6.

The chromosomes in each region (A, B and C) were diluted with isolation buffer and centrifuged at 1200 X g for 20 min. The chromosomes were resuspended in 1 ml of isolation buffer and resedimented in a 10–40% sucrose gradient as above. The fractions A, B and C were collected, diluted up to 15 ml with isolation buffer and centrifuged at 1200 X g for 20 min. These chromosomes were then used in gene transfer experiments. Chromosomes were used in gene transfer experiments immediately after isolation.

Chromosomes from CHOP6 cells growing on monolayers were obtained in a similar way; the final yield of chromosomes, however, was lower, mainly because the percentage of detached cells, as well as the percentage of mitotically arrested cells, in the population was lower. Only 15–20% cell equivalents of chromosomes were recovered.

Transfer of Chromosomes

Pelleted chromosomes were washed and resuspended in HEPES adsorption buffer (pH 7.1) containing 8.0 g/l NaCl, 0.37 g/l KCl, 0.125 g/l $Na_2HPO_4 \cdot 2H_2O$, 1.0 g/l dextrose and 5.0 g/l HEPES (Graham and Van Der Eb, 1973) at a concentration of approximately 2.5 X 10^6 cell equivalent chromosomes per ml, which corresponds to 20 μg/ml of DNA. DNA was measured using the diphenylamine test with calf thymus DNA as the standard (Shatkin, 1969). 2 M $CaCl_2$ were then added to a final concentration of 125 mM, and the chromosome-calcium mixture was left at room temperature for 30 min. Growth medium was removed from recipient cell monolayers in a 100 mm dish, and 2 ml of the chromosome suspension were added to monolayers (2 X 10^6 cells per 100 mm plate) that had been pretreated for 5 min at 37°C with 3 ml α-medium containing 10 μg/ml colchicine, 5 μg/ml colcemid and 2 μg/ml cytochalasin D (Farber and Eberle, 1976). This method gave 2–3 times higher transfer frequencies than the conventional method using poly(L)-ornithine during adsorption of chromosomes (McBride and Ozer, 1973; Willecke and Ruddle, 1975). Control cultures were treated in a similar way, except for the addition of chromosomes. Gentamycin (50 μg/ml) was added during the process of chromosome transfer to prevent mycoplasma contamination (Willecke and Ruddle, 1975; Degnen et al., 1976). Adsorption was carried out at room temperature for 30 min. After adsorption, α complete medium was added to the cells after removal of the inoculum. The cells were then incubated for 2 days at 37°C to allow phenotypic expression of the transferred markers. Next the cells were trypsinized, counted (about 6 X 10^6 cells per plate) and plated at a concentration of 1 X 10^5 cells per 100 mm diameter agar plate as described (Kuroki, 1975). The cells were incubated at 37°C for 10–15 days before colonies were cultured or isolated.

Assay of Agglutination

Cells were grown as monolayers in α-medium containing 10% FCS. The cell monolayers were washed twice with Ca- and Mg-free PBS, and removed from the dish with a solution of 0.02% dissodium versenate in 8.0 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4 and 0.02 g KH_2PO_4 per 1000 ml distilled water (Inbar and Sachs, 1969). They were washed with Ca- and Mg-free PBS and diluted at a concentration of 5–10 X 10^5 cells per ml in the same buffer. To test for agglutination, 0.5 ml of different concentrations of ConA diluted in distilled water were mixed with 0.5 ml of the cell suspension in a 16 mm well of a 24-well Linbro tray, and agglutination was observed at 37°C. At various time intervals, cells were fixed with 4% formaldehyde in PBS, and the presence of agglutinated cells was observed with a phase microscope. A scale of 0 to ++++ was used to estimate the degree of clumping.

Test for Tumorigenicity

Cultures were trypsinized and suspended in complete medium at varying concentrations of cells. Aliquots of 0.1–0.2 ml were injected subcutaneously into nonimmunosuppressed neonatal littermates (1–3 days old) of Syrian or Chinese hamsters. All animals were checked weekly for the appearance of palpable tumors.

Assay for Reverse Transcriptase

Supernatant reverse transcriptase activity was assayed as described (Lieber et al., 1973).

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