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The Relationship between Transformation and Somatic Mutation in Human and Chinese Hamster Cells

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Summary

The frequencies of transformation of primary human and Chinese hamster fibroblasts have been compared with the spontaneous and induced frequencies of mutation for resistance to thioguanine and ouabain, and for ability to use fructose, using the carcinogens benzo (α) pyrene and urethane. Whereas the rates and frequencies of mutation were similar in the two cell systems, transformations to morphologically altered cells was observed only in hamster cells. The frequency of this latter transformation event in hamster cells was about 10^3 greater than the frequencies of mutation in these cells.

The morphologically altered cells formed in the above transformation process cannot grow in agar (*aga*$^-$) and do not produce tumors when injected into animals. The frequency of transition of these latter cells to *aga*$^+$ cells which produce tumors in animals is similar to the mutation-like events.

Introduction

Considerable evidence is now available which indicates that somatic mutation-like events are involved in the process of carcinogenesis. Knudson and his group have provided data which indicate that two mutational events are involved in the development of retinoblastoma (Knudson 1971; Knudson, Hethcote and Brown, 1975), and a genetic etiology has been postulated for a number of other tumors (Knudson, 1973, 1977). Work by Swift indicates that the incidence of cancer measured in heterozygotes carrying the genes for Fanconi anemia (Swift, 1971) and ataxia telangiectasia (Swift et al., 1976) may be elevated. Finally, several observations show that a large number of mutagens are carcinogenic in vivo (Heidelberger, 1975), and that a number of carcinogens are mutagens (Ames et al., 1973). In our own laboratory, we have obtained evidence of a different nature by examining the ability of metaphase chromosomes to transfer cancer phenotypes in hamster cells in vitro (Spandidos and Siminovitch, 1977c, 1977d, 1978; also manuscript submitted). We have found that the ability to transfer these properties was associated with one chromosome size class, and that the frequencies of the chromosome transfer events were similar to the frequencies we had previously demonstrated for single gene markers, such as resistance to methotrexate and ouabain, and prototrophy for glycine, and glycine, adenosine and thymidine (Spandidos and Siminovitch, 1977a, 1977b).

One approach in examining the relationship between mutagenesis and carcinogenesis has been to compare the relative frequencies of the two events in vitro. In Syrian hamster cells, the frequencies of transformation (Berwald and Sachs, 1965; Huberman and Sachs, 1966; Di Paolo, Donovan and Nelson, 1969; Borek and Hall, 1973) are found to be much higher than the frequencies of mutation (Huberman and Sachs, 1976; Ts'o, 1977; Ts'o, Barrett and Myozis, 1977). Similar studies have not been carried out in human cells, although the evidence in the literature indicates that frequencies of transformation in human cells are relatively low (Benedict et al., 1975; Igel et al., 1975; Azzarone, Pedulla and Romanzi, 1976).

Because of the presumed relationship between carcinogenesis and mutagenesis, this raises the question as to whether the low transformability of human cells is reflected in a decreased susceptibility to mutation. In the work described in this paper, we have compared the frequencies of spontaneous and induced mutation for three markers with the frequencies of transformation in Chinese hamster and human cells using two carcinogens, benzo(α)pyrene (B(α)P) and urethane. The results indicate that whereas the frequencies of formation of transformed colonies are much higher in hamster compared with human cells, the spontaneous as well as induced mutation frequencies are similar in the two types of cells.

Results

Frequencies of Transformation of Hamster and Human Cell Strains

Before attempting a comparison of mutation and transformation efficiencies in our systems, it was first important to determine the optimal drug concentrations and times of treatment required for optimal transformation frequencies. The data for both benzo(α)pyrene and urethane with Chinese hamster lung cells using the morphological transformation assay described in Experimental Procedures are shown in Tables 1 and 2. As may be seen, with B(α)P, maximum efficiency is obtained with treatment for 24 hr and 1.0×10^{-6} M of the drug, and with urethane, treatment for 24 hr at 1.1×10^{-2} M produced maximum values. We examined these parameters with B(α)P with human cells as well, but no transformed colonies were observed under any conditions (see Table 1). The comparative frequencies of transformation of primary human

Table 1. Effect of B(α)P Concentration and Duration of Treatment on Transformation of HL and CHL Cells

Treatment		HL Cells				CHL Cells			
B(α)P × 10^{-6} M	Duration (Hr)	Survival (%)	Morphologically Transformed Colonies[a]	Surviving Colonies[b]	Transformation Frequency[c] × 10^{-3}	Survival (%)	Morphologically Transformed Colonies[a]	Surviving Colonies[b]	Transformation Frequency[c] × 10^{-3}
2.0	24	9	0	1023	<1.0	14	12	1430	8.4
1.0	24	22	0	1003	<1.0	25	16	1738	9.2
0.5	24	37	0	1018	<1.0	43	7	1632	4.3
0.1	24	66	0	1052	<1.0	54	0	1053	<1.0
0.01	24	98	0	1043	<1.0	96	0	1035	<1.0
None		100	0	1020	<1.0	100	0	1008	<1.0
10	1	94	0	1061	<1.0	92	0	1023	<1.0
10	5					75	3	1431	2.1
10	10	36	0	1007	<1.0	42	8	1485	5.4
10	48	12	0	1004	<1.0	10	15	1613	9.3
10	96	5	0	1101	<1.0				

[a] Normal transformed colonies display an organized parallel arranagement of cells with very little piling up. Morphologically transformed colonies exhibit a random orientation growth pattern with increased number of multinucleated giant cells and extensive piling up.
[b] Each 100 mm plate usually contained 100–300 colonies.
[c] Transformation frequency = morphologically transformed colonies/total surviving colonies.

and hamster cells using the carcinogens B(α)P and urethane under the optimal conditions for hamster cells are shown in Table 3. The frequencies of transformation of primary hamster lung cultures were 6–9 $\times$ 10^{-3} and 1–2 $\times$ 10^{-3} for B(α)P and urethane, respectively. The morphologies of representative transformed colonies and of normal cells are shown in Figure 1. These colonies maintained their transformed phenotype on subculture. In contrast, we have not observed a single transformed colony among approximately 20,000 colonies of human lung cells treated in a similar manner. The absence of transformed colonies in the human cells does not seem to be due to an extended expression time, since we have followed carcinogen-treated human cells until they senesced without observing evidence for transformation. In confirmation of results in other laboratories, there is therefore a striking difference in susceptibility to transformation in vitro between human and hamster cells. The differences in transformation frequencies of human and hamster cells are unlikely to be due to differences in activation of the carcinogens, since first, urethane does not require such activation to act as a carcinogen (Kaye and Trainin, 1966); second, the survival of both types of cells in the presence of the drugs is similar (see Tables 4–6); and third, we have found, using a method described earlier (Huberman, Selkirk, and Heidelberger, 1971), that B(α)P forms similar amounts of water-soluble products in our human and hamster lines and strains.

Frequencies of Spontaneous and Induced Mutation of Human and Hamster Cell Strains

To compare the mutation frequencies of the human and hamster cell strains, we chose three markers for which there was evidence that mutation could be demonstrated in primary human fibroblasts. Mutants resistant to thioguanine (*thg*^{r}), which involve the enzyme hypoxanthine guanine phosphoribosyl transferase, can be isolated in human cells, and both the frequencies and rates of the mutation have been measured (Cox and Masson, 1976a, 1976b). Resistance to ouabain (*oua*^{R}), which involves the membrane-associated, Na^+/K^+-activated

Table 2. Effect of Urethane Concentration and Duration of Treatment on Transformation of CHL Cells

Treatment					
Urethane (M)	Duration (Hr)	Survival (%)	Morphologically Transformed Colonies[a]	Surviving Colonies[b]	Transformation Frequency[c] $\times$ 10^{-3}
1.5×10^{-2}	24	43	5	2503	2.0
1.1×10^{-2}	24	62	6	2895	2.1
1.1×10^{-3}	24	78	0	1031	<1.0
1.1×10^{-4}	24	95	0	1010	<1.0
None		100	0	1033	<1.0
1.1×10^{-2}	5	88	0	1042	<1.0
1.1×10^{-2}	48	54	3	1763	1.7
1.1×10^{-2}	96	21	3	1655	1.8

[a, b, c] As described in Table 1.

Table 3. Frequencies of Morphological Transformation of Human Lung and Chinese Hamster Lung Cell Strains Induced by B(α)P or Urethane after a 24 Hr Exposure

Cell Strain	Carcinogen	Total Morphologically Transformed Colonies[a] (Colonies per Experiment)	Total Surviving Colonies[b] (Colonies per Experiment)	Average Transformation Frequency[c] $\times$ 10^{-3} (Frequency per Experiment)
HL	None	0 (0, 0, 0)	6400 (1762, 1984, 2654)	<0.2 (<0.6, <0.6, <0.4)
HL	B(α)P[d]	0 (0, 0, 0)	6632 (1856, 2562, 2214)	<0.2 (<0.6, <0.4, <0.5)
HL	Urethane[e]	0 (0, 0, 0)	6898 (2350, 1910, 2638)	<0.2 (<0.5, <0.6, <0.4)
CHL	None	0 (0, 0, 0)	5379 (1581, 1312, 2486)	<0.2 (<0.6, <0.7, <0.4)
CHL	B(α)P	42 (15, 8, 19)	5298 (1643, 1230, 2425)	8.1 (9.1, 6.5, 7.8)
CHL	Urethane	11 (3, 2, 6)	6386 (1922 1810, 2654)	1.7 (1.5, 1.1, 2.2)

[a, b, c] As described in Table 1.
[d] B(α)P = 1.0×10^{-6} M.
[e] Urethane = 1.1×10^{-2} M.

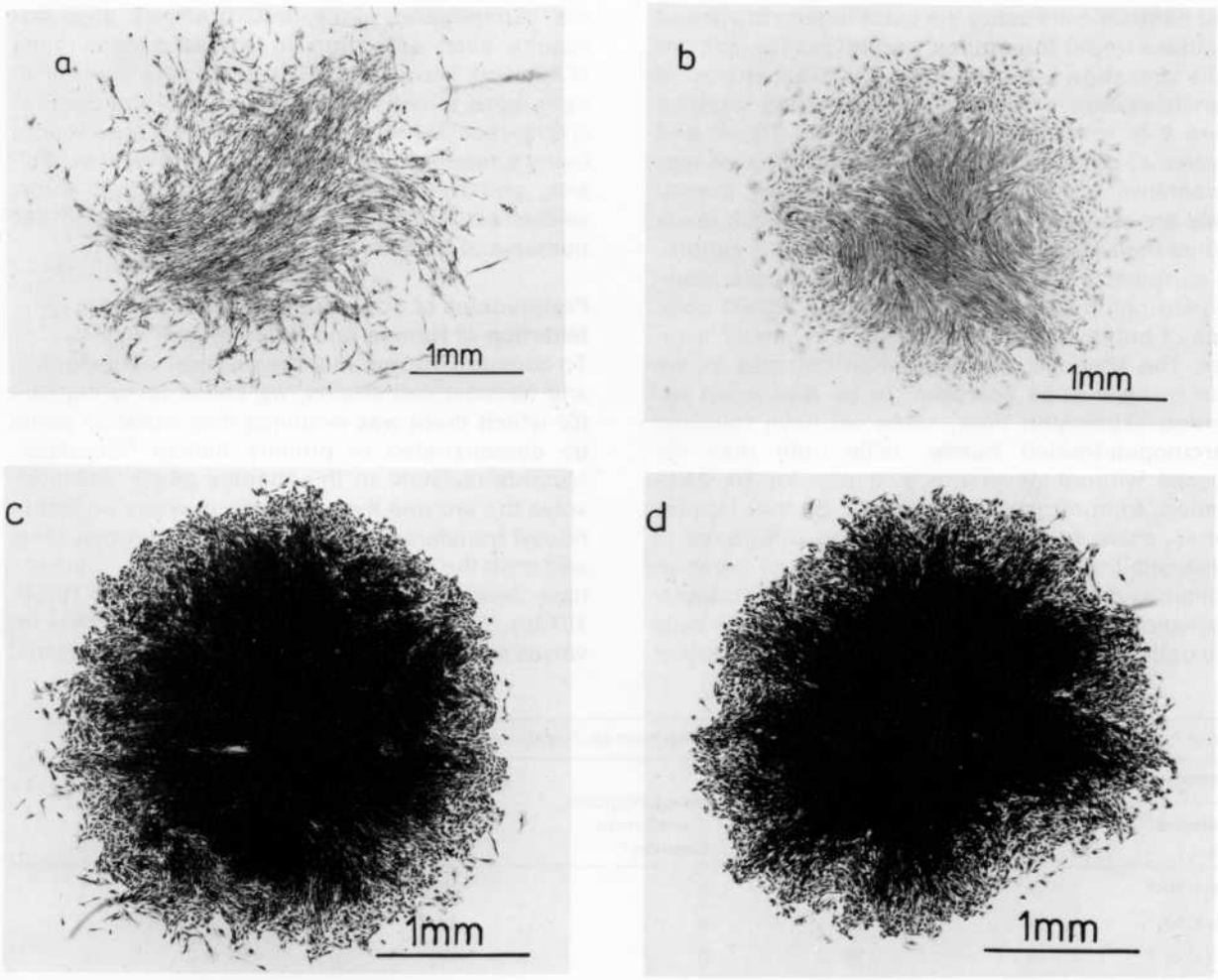

Figure 1. Normal and Transformed Colonies

Normal: (a) HL cells; (b) CHL cells. Morphologically transformed CHL cells: (c) with B(α)P; (d) with urethane.

ATPase (Baker et al., 1974), acts co-dominantly, and the feasibility of using this marker for genetic studies in human fibroblasts has been demonstrated (Mankovitz, Buchwald and Baker, 1974). The observation that mutations involving the ability to use fructose (*fru*$^+$) can be isolated in human fibroblasts is very recent, and evidence on the nature of the genetic change is therefore less developed. The fact that the frequencies are of the same order as for other mutations and are increased by x-rays argues for their status as true mutants (Cox and Masson, 1974).

In comparing mutation frequencies between cell strains, it is of course important to know whether expression times are similar in both cases. Since much of the data described in this paper was obtained with the *thg*^{r} marker, we first examined the relative expression times for thioguanine resistance in human and hamster cells after treatment with B(α)P. Cells were treated with the carcinogen for 24 hr at 37°C, and then selection was effected for thioguanine resistance at various times thereafter. As may be seen in Figure 2, for both cell strains, the frequency of mutants began to increase between 2 and 4 days, and reached a maximum between 8 and 10 days, as was found earlier by Cox and Masson (1976) for human fibroblasts after x-ray treatment. This result indicated that expression times were similar for human and hamster cells for this marker. The time required for expression of ouabain resistance was found to be shorter (4–5 doublings) in earlier work (Mankovitz et al., 1974), and we have confirmed these results. Although the previous studies indicated that no expression time was required for the *fru*$^+$ marker (Cox and Masson, 1974), the cells were allowed to grow for a week post-treatment (4–5 doublings) before selection was applied.

It is also important in experiments of this kind to ensure that all selected colonies breed true and do

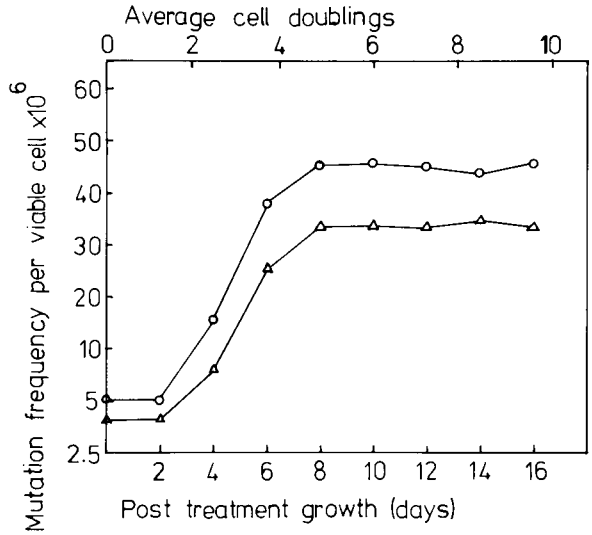

Figure 2. Relationship between Duration of Post-Treatment Growth and Induced Mutation Frequency to Thioguanine Resistance

Human lung (○) and Chinese hamster lung (△) cells after treatment with 1.0×10^{-6} M B(α)P for 24 hr at 37°C. Mutation frequencies in untreated controls varied between $3\text{-}6 \times 10^{-6}$ mutations per viable cell.

not represent cells which have survived due to cross-feeding or to some other transitory phenomenon. In preliminary tests for *thg*^{r} and *oua*^{R} in CHL cells, we found that 10/10 and 8/8 colonies, respectively, bred true. Similar results were found with human cells for these markers. We also tested a large number of the resistant colonies obtained in the mutagenesis experiments, and all of them bred true for *oua*^{R} and *thg*^{r}. Five each of the human and hamster *thg*^{r} lines obtained in the above experiments were tested for hprt$^-$ activity and were found to be negative (<0.2% of wild-type activity). For the *fru*$^+$ phenotype, in preliminary tests, we found that only 5/10 CHL colonies tested were fructose-positive on retesting, and HF behaved similarly. In this system, therefore, *all* colonies obtained in the mutagenesis experiments were tested for their *fru*$^+$ phenotype before inclusion in the data. The results for the frequencies of thioguanine resistance in human and hamster lung cells are shown in Table 4. As may be seen, normal and induced mutation frequencies were similar for the two types of cells.

Similar results were obtained with the other two markers. For ouabain resistance (*oua*^{R}), the induced frequencies were again similar, but about 4–5 fold higher for the hamster cells (Table 5). In the case of the *fru*$^+$ marker, the frequencies were again similar in the two cell systems (Table 6). The general conclusion from these results is that the frequencies of spontaneous and induced mutation are similar in primary human and hamster cells.

Rates of Mutation in Human and Hamster Cells

Comparison of spontaneous frequencies of mutation are open to doubt because of their dependence on previous culture history. In our experiments, we sought to minimize this problem by using early passage cells. Nevertheless, to obtain a more meaningful measurement of mutation susceptibility, we measured the mutation rates to thioguanine resistance in the two types of cells by a Luria and Delbrück fluctuation test (Luria and Delbrück, 1943). As may be seen in Table 7, the results of such experiments provide support for the earlier data on spontaneous frequencies in that the rates of mutation were also similar in the human and hamster cells.

Frequency of Spontaneous and Induced Mutation of Transformed Hamster Cells to the *Aga*$^+$ Phenotype

Comparison of the data in Table 3 with Tables 4–6 shows that the frequency of transformation in hamster cells is much higher than the frequency of mutation. This result is similar to that found by others investigators in Syrian hamsters (Huberman and Sachs, 1976; Ts'o, 1977; Ts'o et al., 1977). The phenotype being measured in those experiments is a morphological alteration of the cell and is probably similar to that observed on spontaneous transformation of hamster cells. It is also similar to that observed when primary hamster cells are rescued from senescence by metaphase chromosomes obtained from the Chinese hamster ovary (CHO) line (Spandidos and Siminovitch, 1978). Although such cells are morphologically transformed, they usually do not show anchorage independence, as measured by growth in agar (*aga*$^-$), and do not produce tumors when injected into animals. They probably represent an early stage in the carcinogenesis process in hamster cells, since they can be converted into cells which are anchorage-independent (*aga*$^+$) and which produce tumors in animals, either by transfer of metaphase chromosomes or spontaneously (see below).

It was next of interest to determine whether the frequency of this latter alteration from *aga*$^-$-transformed cells to *aga*$^+$ cells was similar to that characteristic of the first transformation event or, in contrast, of the mutation-like events described in Tables 4–7.

We first examined the ability of our chemically transformed hamster lines to give rise to *aga*$^+$ cells by growing the cells and plating on agar at various times. As may be seen in Table 8, none of the transformed hamster clones produced measurable *aga*$^+$ colonies shortly after transformation. On subsequent growth, however, all the cultures gave rise

Table 4. Frequency of Spontaneous and Carcinogen-Induced Mutation to *thg*^{r} in Diploid Human and Chinese Hamster Lung Cells[a]

Cell Strain	Carcinogen	Concentration (M)	Survival (%)	Expression Time: Doublings	Expression Time: Days	PE (%)	Total Number of Viable Cells × 10^6	Number of *thg*^{r} Colonies	Mutation Frequency × 10^{-6}	Induced Mutation Frequency × 10^{-6}
HL	None		100	9	10	18	1.00[b]	5	5.0	
HL	B(α)P	1.0 × 10^{-6}	22	6	10	11	0.15	7	46.6	41.6
HL	Urethane	1.1 × 10^{-2}	53	7	10	16	0.45	6	13.3	8.3
CHL	None		100	10	10	15	1.70	7	4.1	
CHL	B(α)P	1.0 × 10^{-6}	25	7	10	10	0.24	8	33.3	29.2
CHL	Urethane	1.1 × 10^{-2}	61	8	10	12	0.71	10	14.1	10.0

[a] Selection of *thg*^{r} colonies was effected in α-medium containing 15% DFCS and 1.5 × 10^{-5} M thioguanine. 1 × 10^5 cells (not corrected for PE) per 100 mm plate were incubated for 2–3 weeks at 37°C without change of the medium (20 ml per plate).
[b] These numbers were derived by multiplying the total number of cells plated by the plating efficiency (PE).

Table 5. Frequency of Spontaneous and Carcinogen-Induced Mutation to *oua*^{R} in Diploid Human and Chinese Hamster Lung Cells[a]

Cell Strain	Carcinogen	Concentration (M)	Survival (%)	Expression Time: Doublings	Expression Time: Days	PE (%)	Number of Viable Cells × 10^7	Number of *oua*^{R} Colonies	Mutation Frequency × 10^{-7}	Induced Mutation Frequency × 10^{-7}
HL	None		100	6	7	17	4.00[b]	2	0.5	
HL	B(α)P	1.0 × 10^{-6}	25	4	7	10	0.80	4	5.0	4.5
HL	Urethane	1.1 × 10^{-2}	51	5	7	12	1.43	2	1.4	0.9
CHL	None		100	7	7	16	0.95	2	2.1	
CHL	B(α)P	1.0 × 10^{-6}	23	5	7	10	0.23	5	22.0	19.9
CHL	Urethane	1.1 × 10^{-2}	56	6	7	13	0.43	3	7.1	5.0

[a] Selection of *oua*^{R} colonies was effected in α-medium containing 15% FCS and 1 × 10^{-6} M ouabain (for human cells) or 3 × 10^{-3} M (for hamster cells). 1 × 10^6 cells (not corrected for PE) per 100 mm plate were incubated for 2–3 weeks at 37°C without change of the medium (20 ml per plate).
[b] As in Table 2.

Table 6. Frequency of Spontaneous and Carcinogen-Induced Mutation to *fru*$^+$ in Diploid Human and Chinese Hamster Lung Cells[a]

Cell Strain	Carcinogen	Concentration (M)	Survival (%)	Expression Time: Doublings	Expression Time: Days	PE (%)	Number of Viable Cells × 10^6	Number of *fru*$^+$ Colonies	Mutation Frequency × 10^{-6}	Induced Mutation Frequency × 10^{-6}
HL	None		100	6	7	20	6.36[b]	7[c]	1.1	
HL	B(α)P	1.0 × 10^{-6}	18	4	7	12	0.73	11	15.0	13.9
HL	Urethane	1.1 × 10^{-2}	47	5	7	15	0.75	16	8.0	6.9
CHL	None		100	7	7	14	1.50	3	2.0	
CHL	B(α)P	1.0 × 10^{-6}	23	5	7	9	0.16	4	24.0	22.0
CHL	Urethane	1.1 × 10^{-2}	54	6	7	11	0.66	5	7.6	5.6

[a] Selection of *fru*$^+$ colonies was effected in a α-medium lacking glucose and containing 15% DFCS and 1 mg/ml βD(−)fructose. 2 × 10^5 cells (not corrected for PE) per 100 mm plate were incubated for 2–3 weeks at 37°C without change of the medium (20 ml per plate).
[b] As in Table 2.
[c] The numbers in this column represent only those colonies which showed a *fru*$^+$ phenotype on retesting.

to *aga*$^+$ colonies. As may also be seen in Table 8, concomitant with the appearance of the *aga*$^+$ phenotype, the cultures also acquired the ability to produce tumors in animals during growth in vitro. Cells derived from these tumors showed the *aga*$^+$ phenotype and were able to grow in suspension. Since we have never obtained *aga*$^+$ cells directly from primary CHL cells, even with >10^8 cells at

Table 7. Fluctuation Analysis of the Rate of Mutation to *thg*^{r} in Human and Hamster Lung Cells

	Human	Hamster
Number of Replicate Cultures	25	25
Initial Number of Viable Cells per culture	100	100
Final Number of Viable Cells per Culture	4×10^6	4×10^6
N = 0	8	5
1	6	7
2	4	5
3	2	2
4	1	1
5	1	
6		1
7	1	
8		1
10	1	
11		1
13		1
15	1	
16		1
Average *thg*^{r} Colonies per Culture	2.1	3.2
Variance	12.8	18.5
Fraction of Cultures with Zero *thg*^{r} Colonies	0.32	0.20
Mutation Rate	1.9×10^{-7}	2.8×10^{-7}

The experiment was started by inoculating 25 replicate cultures (10 ml) with approximately 100 viable cells each in 25 cm^2 flasks. After 3 weeks of growth at 37°C, the cells were transferred into 75 cm^2 flasks. A week later, the cells in each culture were trypsinized, and 4×10^6 viable cells were plated in α-medium containing 15% DFCS and 1.5×10^{-5} M 6-thioguanine. 1×10^5 cells per 100 mm plate were incubated for 2–3 weeks at 37°C without change of the medium (20 ml per plate). The mutation rate was calculated by the P_0 method as described (Luria and Delbrück, 1943).

risk. These results provided further evidence for the concept that morphologically transformed colonies represent a step in the progression from normal to fully neoplastic cells.

To examine the change from transformed *aga*$^-$ to neoplastic *aga*$^+$ cells in a more quantitative manner, we have measured the frequencies of appearance of *aga*$^+$ colonies in a single step with and without treatment by carcinogens. For comparative purposes, three different types of transformed *aga*$^-$ cells were used for this purpose: CHLBP1 were cells transformed by B(α)P; CHLTF1 were cells which transformed spontaneously; and CHLRFStran4 were cells derived by chromosome transfer from CHO cells into senescent primary Chinese hamster lung cells. There does not appear to be any expression time needed for the *aga*$^+$ phenotype, since as shown in Table 9, we found no increase in *aga*$^+$ colonies when selection was applied 0–21 days. As this table also shows, no *aga*$^+$ colonies were obtained with either primary CHL or HL cells even with long expression times. At least three conclusions can be drawn from the results shown in Table 10. First, the three types of transformed cultures were able to give rise to *aga*$^+$ colonies, and the frequencies were similar for all of them with and without carcinogen treatment. Second, both B(α)P and urethane acted as mutagens in this system and were able to increase the frequencies of the *aga*$^-$ to *aga*$^+$ event. Finally, comparison of the data in Table 10 with Tables 3–7 shows that the frequency of the *aga*$^-$ to *aga*$^+$ transition is similar to that found when biochemical mutants are selected in primary hamster and human cells, rather than the frequency found for the transformation of normal hamster cells into morphologically transformed cells. It is of interest that the frequencies for the *aga*$^-$ to *aga*$^+$ transition found in our experiments are similar to those observed earlier for the same conversion in BHK cells treated with nitrosomethylurea and 4-nitroquinoline-1-oxide (Bouck and Di Mayorca, 1976).

Discussion

The experiments described in this paper represent the first attempt to undertake a quantitative comparative study of mutagenesis, transformation and carcinogenesis in two types of cells in vitro. A number of conclusions can be drawn from our results. First, the frequency of transformation in primary Chinese hamster cells is about 10^3 greater than the frequency of mutation at single loci. This observation is similar to that found by other investigators in Syrian hamster cells (Huberman and Sachs, 1966, 1976; Ts'o, 1977; Ts'o et al., 1977). Second, as shown in earlier investigations, such transformed cells represent an early stage in progression in vitro to cells capable of producing tumors in animals (Kuroki and Sato, 1968; Lasne, Gentil and Chouroulinkov, 1974). In our own laboratory, we have found that *aga*$^+$ colonies cannot be produced from normal cells by a single chromosomal transfer event (Spandidos and Siminovitch, 1977d), and that no *aga*$^+$ colonies are observed after treatment of normal cells with carcinogens. The first stage transformation event therefore seems to result in morphologically transformed cells which cannot produce colonies in agar (*aga*$^-$) and which do not produce tumors when injected into animals. The frequency of transition from these first stage transformed cells to anchorage-independent and malignant cells (*aga*$^-$ to *aga*$^+$) is similar to the frequency observed for mutation for

Table 8. Development of the *aga*$^+$ Phenotype (and Tumorigenicity) by Chinese Hamster Lung Cells Transformed with B(α)P or Urethane

	Ratio of Plating Efficiencies (Agar Plate[a]/Plastic Plate[b])				
	Months in Culture/Approximate Cell Doublings				
Cell Line	1/20	2/45	3/70	4/100	5/130
CHL-BP1[c]	$<5 \times 10^{-7}$ (0/3)[d]	$<5 \times 10^{-7}$	1×10^{-7}	3×10^{-6}	5×10^{-5} (2/3)
CHL-BP2	$<5 \times 10^{-7}$ (0/3)	2×10^{-7}	5×10^{-6}	1×10^{-5}	8×10^{-5} (3/3)
CHL-UR1	$<5 \times 10^{-7}$ (0/3)	6×10^{-6}	1×10^{-5}	8×10^{-5}	5×10^{-4} (3/3)
CHL-UR2	$<5 \times 10^{-7}$ (0/3)	$<5 \times 10^{-7}$	$<5 \times 10^{-7}$	1×10^{-7}	8×10^{-6} (1/3)

[a] α-Medium containing 1% Noble agar and 10% FCS.
[b] α-Medium containing 10% FCS.
[c] CHL-BP1 and CHL-BP2 represent two clones of cells transformed by B(α)P, and CHL-UR1 and CHL-UR2 represent two clones of cells transformed by urethane.
[d] The numbers in parentheses represent the ratio of animals with tumors per animal tested 2 months after subcutaneous injection of 30 day old nude mice with 1×10^6 cells.

Table 9. Frequency of B(α)P Induced Mutation to *aga*$^+$ in HL and CHL Cell Strains and in the CHL-BP1 Cell Line

Cell Strain or Line	Post-Treatment Growth[a]	Number of Viable Cells $\times 10^6$	Number of *aga*$^+$ Colonies[b]	Mutation Frequency $\times 10^{-7}$
HL	0	12	0	<0.8
HL	7	15	0	<0.6
HL	21	25	0	<0.4
HL	30	28	0	<0.4
CHL	0	10	0	<1.0
CHL	7	22	0	<0.5
CHL	21	28	0	<0.4
CHL-BP1	0	2.9	22	75.8
CHL-BP1	2	3.0	25	83.3
CHL-BP1	7	5.1	34	66.6
CHL-BP1	14	2.8	20	71.4
CHL-BP1	21	2.1	18	85.7

[a] Cells were treated with 1.0×10^{-6} M B(α)P for 24 hr at 37°C.
[b] Selection of *aga*$^+$ colonies was effected in α-medium containing 1% Noble agar and 10% FCS. 2×10^5 cells per 100 mm plate were incubated for 2–3 weeks at 37°C.

three independent loci in primary hamster and human cells. Third, although there is a great difference in susceptibility to transformation between human and hamster cells, the frequencies of spontaneous and induced mutation are of the same order of magnitude in the two cell types.

With respect to the latter conclusion, we recognize that for the induced frequencies, we have only compared the efficiencies at one drug dose. Although this dose was on the linear range of frequency versus concentration curve as determined in preliminary experiments, it represents a possible weakness in the data. This criticism, however, does not apply to the spontaneous frequencies and rates where the values were similar in the two cell types. Furthermore, the major conclusion we wish to derive from our data is that mutation frequencies between human and hamster cells are not grossly different in comparison with transformation frequencies, and we believe that this view is supported by the results given in Tables 3–7. It is difficult to assess quantitatively the magnitude of the difference in susceptibility to transformation between human and hamster cells, since no transformed colonies were observed in the human cells. In the only other published studies on chemical transformation of human diploid cells, the investigators were also unsuccessful in obtaining transformed cells in normal human cell cultures, but no quantitative data were presented on the lowest level of detection (Igel et al., 1975). In our own laboratory, we have found on two occasions that 1

Table 10. Frequency of Spontaneous and Carcinogen-Induced Mutation to *aga*$^+$ in Chinese Hamster Cell Lines[a]

Cell Line	Carcinogen	Concentration (M)	Survival (%)	Expression Time		PE (%)	Number of Viable Cells $\times 10^6$	Number of *aga*$^+$ Colonies	Mutation Frequency $\times 10^{-7}$	Induced Mutation Frequency $\times 10^{-7}$
				Doublings	Days					
CHL-BP1	None		100	7	7	50	8.00	4	5.0	
CHL-BP1	B(α)P	1.0×10^{-6}	31	6	7	23	3.45	25	72.4	67.4
CHL-BP1	Urethane	1.1×10^{-2}	60	6	7	32	5.91	12	20.3	15.3
CHLTF1	None		100	7	7	44	20.8	5	2.4	
CHLTF1	B(α)P	1.0×10^{-6}	23	5	7	21	3.98	16	40.2	37.8
CHLTF1	Urethane	1.1×10^{-2}	48	6	7	30	7.76	8	10.3	7.9
CHLRFStran4	None		100	7	7	35	20.0	3	1.5	
CHLRFStran4	B(α)P	1.0×10^{-6}	25	5	7	18	5.36	12	22.4	20.1
CHLRFStran4	Urethane	1.1×10^{-2}	52	6	7	23	6.97	6	8.6	7.1

[a] Selection of *aga*$^+$ colonies was effected as described in Table 9.

$\times 10^7$ human diploid fibroblast cells treated with B(α)P at about the fiftieth generation went on to senescence normally after 15–20 further generations without the appearance of any visible transformed colonies. Moreover, all *thg*^{r}, *oua*^{R} and *fru*$^+$ mutants isolated in these studies and tested were found to be susceptible to senescence. These observations, plus the general difficulty of obtaining chemically transformed human lines, indicate that the difference in susceptibility between human and hamster cell lines is indeed very large.

Primary human fibroblasts can be transformed with viruses, particularly SV40, but this fact may not be relevant in the present context. In the case of virus transformation, the appropriate genetic material is presumably introduced with the virus, whereas in chemical transformation, the initial event occurs in the cell.

Thus whereas human cells are highly refractory to transformation with carcinogens, they show normal frequencies and rates of mutation. They also seem to show normal susceptibility to the same carcinogens in respect to mutagenesis. These results could be explained by assuming that transformation resulted from the action of one metabolite of benzo(α)pyrene, and mutation by another. Alternatively, the differences could be due to diverse mechanisms of action of the same metabolite or different efficiencies of repair for transformation in human and cancer cells. None of these hypotheses, however, seems particularly attractive because of the magnitude of the differences in transformation efficiencies and because of the qualitative correlations observed frequently in the past between the ability of a substance to act as a carcinogen and as a mutagen.

One explanation for the low comparative susceptibility of human cells may lie in the number of steps required to produce a transformed cell. According to this hypothesis, one step would be required to form first stage hamster transformants, and two or more could be required to reach the same stage in human cells. Our experiments on chromosome transfer are perhaps of interest in this regard. Primary hamster cells can be rescued from senescence and transformed in the process by transfer of metaphase chromosomes from transformed cells (Spandidos and Siminovitch, 1978). It seems probable that this transformation process involves the transfer of a single gene via the donor chromosomes. So far such experiments have failed in our hands in the human system (D. A. Spandidos and L. Siminovitch, unpublished results). This lack of success might be attributed to the need for two or more genetic events to produce a human transformed cell. The idea that several steps are involved in human carcinogenesis in vivo has been proposed previously by other investigators (Beren-

blum, 1941; Armitage and Doll, 1957; Ashley, 1969). It is also possible that in order to observe a morphological transformation, a certain threshold must be reached, and that hamster cells are inherently closer than human cells to that threshold. Under these circumstances, hamster cells would be more susceptible to transformation in vitro.

The high susceptibility of hamster cells to transformation with carcinogens as compared with spontaneous or induced mutation on the one hand, and with the susceptibility of human cells to transformation on the other hand, might be due to differences in number of targets. According to this hypothesis, hamster cells would contain multiple targets capable of being altered to produce a transformed phenotype or, stated differently, many different alterations could produce the transformed phenotype in such cells. In this case, the frequency of transformation would be greater than that for spontaneous or induced mutation involving a single gene locus, and greater than in human cells, if the latter cells are assumed to have a limited number of targets. Furthermore, although the first and second steps of carcinogenesis in vitro in the hamster might appear to be quite distinct because of differences in their frequencies, the distinction could simply be due to numbers of targets or differences in the number of changes which could produce the transformed phenotype. According to this view, once the primary event had occurred, the secondary event could only take place in this one target, and thus its frequency would be comparatively low.

There is very little evidence for any of these views. We also do not know whether hamster cells are particularly sensitive to transformation or whether human cells are especially refractory. Experiments are under way to examine this question. In human cells, recent data indicate that fibroblasts from patients with hereditary adenomatosis of the colon show increased susceptibility to transformation with Kirsten murine sarcoma virus (Pfeffer and Kopelovich, 1977). Cells from such patients, as well as from patients with other syndromes involving increased cancer susceptibility, may provide useful material for examining some of the above hypotheses.

Experimental Procedures

Cells and Growth Medium

The human female fetal lung (HL) cells were provided by Dr. M. Buchwald at their second generation and were grown in α-MEM (Stanners, Elicieri and Green, 1971) containing 15% FCS. The Chinese hamster lung (CHL) cells were derived from explants of female hamsters and were grown under the same conditions.

Mutagenesis

Early passages of HL or CHL cells (P3-P6) were trypsinized, resuspended in α-MEM containing 15% FCS and plated at a concentration of 2×10^6 cells per 75 cm^2 flask at 37°C. After 24 hr, the cells were treated with the carcinogen for 24 hr. Benzo(α)pyrene (Sigma) was dissolved in DMSO and diluted into culture medium to the desired final concentration. The final concentration of DMSO was <0.1%. Control cultures were treated with DMSO at the same concentration. Urethane (Sigma) was directly dissolved into culture medium to the desired final concentration. After the treatment, all cultures were washed with phosphate-buffered saline (PBS) and trypsinized; aliquots were taken to determine cell survival and subcultured by 1:4 dilutions in α-MEM containing 15% FCS.

Selection of Mutants

For the thioguanine selection, the cells were grown for 10 days of expression time (time sufficient for more than six doublings) and then replated at a concentration of 1×10^5 cells per 100 mm plate in selective medium. This medium consisted of α-MEM containing 15% dialyzed fetal calf serum (DFCS) and the appropriate concentrations of 6-thioguanine (thg). After 2-3 weeks, plates were scanned for colonies using a microscope, and some of the colonies were picked using a stainless steel cylinder and trypsin, whereas the remaining colonies were fixed with methanol and stained with 10% aqueous Giemsa for counting.

The ouabain selection was carried out as described (Mankovitz et al., 1974). Different concentrations of ouabain were used for the selection with hamster and human cells because of the large differences in susceptibility of these cells to the drug. The expression times used were 7 days (more than four cell doublings). The cells were then subcultured at a concentration of 1×10^6 cells per 100 mm plate into α-MEM containing 15% FCS and ouabain. After growth for 2-3 weeks at 37°C, colonies were picked or stained as above.

The selection for cells able to utilize fructose was similar to that described (Cox and Masson, 1974). The expression times used, however, were 7 days. The cells were then subcultured at a concentration of 2×10^5 cells per 100 mm plate into α-MEM lacking glucose and containing 15% DFCS and 1 mg/ml β D(−) fructose. After growth for 2-3 weeks at 37°C, colonies were picked or stained as above.

Testing for Mutants

To test for 6-thioguanine resistance, colonies were picked and grown in nonselective medium. The cells were then reexposed to thg at the selective concentration and tested for growth in glycine containing HAT medium (containing 1×10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin, 5×10^{-4} M thymidine and 3×10^{-6} glycine). Normal cells can overcome an animopterin block in de novo purine synthesis if hypoxanthine thymidine and glycine are provided. *thg*^{r} mutants, however, are unable to grow in this medium because they cannot utilize hypoxanthine as a sole source of purine. Such cultures were also tested for hprt activity using the assay described (Chasin and Urlaub, 1976).

Cells were tested for ouabain resistance by a cell proliferation assay similar to that described (Mankovitz et al., 1974). Results obtained in this way have been shown to correlate well with Na^+/K^+ ATPase activity. Cells from each colony were transferred to 35 mm plates and cultured in nonselective medium until enough cells were obtained for testing. An equal number of cells were seeded into replicate plates (35 mm) containing different concentrations of ouabain and incubated for 7 days. The plates were then harvested and the cells were counted.

To test for the ability to utilize fructose, the cells from individual colonies were picked and grown in 35 mm plates in nonselective medium. After 7 days, the cells were then retested for their ability to grow in the presence of fructose.

Morphological Transformation Assay

HL or CHL cells were trypsinized, resuspended in α-MEM containing 15% FCS and plated at a concentration of $1\text{-}5 \times 10^3$ cells per

100 mm plate in 20 ml medium. After 24 hr, the appropriate concentration of carcinogen was added and incubation was continued at 37°C for 24 hr. Cells were then washed 4 times with α-MEM containing 10% FCS, and 20 ml of α-MEM containing 15% FCS were added to each plate. After 2-3 weeks of growth at 37°C, colonies were scored for morphological transformation with phase-contrast microscopy. Normal untransformed colonies display an organized parallel arrangement of cells with very little piling up. Morphologically transformed colonies exhibit a random orientation growth pattern with increased number of multinucleated giant cells and extensive piling up. Colonies were picked or stained as described above.

Assay for Anchorage Independence or Ability to Grow on Agar (*aga*$^+$)

This assay for numbers of *aga*$^+$ colonies was performed as described (Kuroki, 1975; Spandidos and Siminovitch, 1977d).

Tumorigenicity Studies

Cultures were trypsinized and suspended in α-MEM at varying concentrations of cells, and 0.2 ml were subcutaneously injected into male nude mice (30 days old).

All animals were checked weekly for the appearance of palpable tumors over a period of 3 months.

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