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Selection of Morphologically Normal Cell Lines from Polyoma-transformed BHK21/13 Hamster Fibroblasts

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A selective method was devised for the isolation of "revertants" from polyoma-transformed sublines derived from BHK21/13 Syrian hamster fibroblasts. A hybrid, polyploid subline was obtained by growing together, in mixed culture in the presence of aminopterin, two variant BHK21/13 sublines lacking either inosinic acid pyrophosphorylase or thymidine kinase. Whereas these variant sublines were resistant to 6-thioguanine or to 5-bromodeoxyuridine, the hybrid had regained sensitivity to both analogues. By plating a polyoma-transformed subline derived from this hybrid in the presence of 6-thioguanine, resistant clones were obtained with a frequency of about 10^{-4}. All of these surviving clones had a reduced chromosome complement and some of them had regained a normal phenotype.

Morphological transformation of hamster cells by polyoma virus in vitro confers on the cell the ability to grow in a critical concentration of agar (14). Although exceptions have been reported (6), transformation as detected in vitro is usually correlated closely with increased transplantability in the animal, i.e., with malignancy. In view of the fact that polyoma-transformed hamster cells contain ribonucleic acid (RNA) molecules complementary to polyoma deoxyribonucleic acid (DNA; 1), it is tempting to assume that the transformed phenotype is entirely and directly controlled by integrated viral genes. Since the transformed phenotype was shown to be dominant in somatic mouse cell hybrids (4), and thus to depend on the addition of genetic information, the inactivation or deletion of such genes might cause reversion to the normal state.

In the present work, attempts were made to obtain revertants from polyoma-transformed polyploid sublines of BHK21/13 hamster fibroblasts by selecting for chromosome loss. The underlying assumption was that a polyploid cell, contrary to a diploid one, may afford extensive chromosome deletion without loss of viability. On the assumption that random loss of chromosomes by transformed polyploid cells may result in the loss of genes controlling the transformed phenotype, a potential method was devised for the selection of chromosome loss. This method was based on the hypothesis that chromosome loss in cultured lines is largely random and often multiple, because it is likely to arise through the occurrence of abnormal mitotic spindles, leading to unequal distribution of chromosomes to the daughter cells (3). Selection for the loss of any specific character would thus result in the deletion of other characters not necessarily linked to the one selected against.

In fact, by this method, a number of sublines which had lost several chromosomes was obtained, and some of these showed normal or quasinormal morphology and reduced plating efficiency in soft agar.

MATERIALS AND METHODS

Cells. All sublines were derived from the line BHK21/13 of Syrian hamster fibroblasts kindly provided for us by M. Stoker (18). The line is kept frozen in liquid nitrogen to prevent any possible modification of its characteristics through the selective growth of altered variants. In our laboratory, it displays typically "normal" morphology (see definition below), and its plating efficiency in soft agar medium is lower than 0.1%.

Growth medium. Reinforced Eagle's medium (20) plus 10% Tryptose Phosphate Broth (Difco) and 10% calf serum was used routinely. The serum concentration was raised to 20% when cells were plated in numbers that would give rise to discrete colonies. Growth medium containing 0.34% agar (soft agar medium) was used for the selective growth of transformed cells (14).

Culture methods. The cells were routinely grown

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in 60-mm tissue culture dishes (Falcon plastic) at 37 C in a humidified atmosphere containing 10% CO_2. Plating efficiency of untreated cells and survival to drug treatments were determined by plating suitable numbers of cells in fluid medium and by counting visible colonies 6 to 8 days later. To determine the frequency of transformed cells in a cell population, the cells were plated in soft agar medium, and visible colonies were counted 8 to 10 days later. Four to eight dishes per point were scored in each case.

Clonal isolation of sublines of normal cells was carried out by trypsinizing, in small siliconized stainless-steel cylinders, selected colonies grown from single cells in fluid medium (17). Clonal isolation of transformed sublines was carried out by selecting colonies grown from single cells in soft agar medium, sucking them in the tip of a Pasteur pipette, and transferring them in culture dishes containing fluid medium. Here the colonies would readily attach to the bottom and undergo massive growth.

Infection with polyoma virus. The small-plaque mutant of polyoma virus was used (stock PySK1). For infection, cell suspensions in buffered saline plus 5% calf serum were mixed with the virus to a final cell concentration of 10^6 cells/ml (400 plaque-forming units per cell). Virus adsorption was carried out for 1 hr at 37 C; a small magnetic stirrer was used to avoid cell clumping. After centrifugation to remove unadsorbed virus, the cells were diluted and plated in soft agar medium.

Chromosome counts. Growing cells were treated for 4 hr with 2 μg (per ml) of Colcemid (CIBA). The arrested metaphases were collected from the monolayer by gentle pipetting; they were then centrifuged and resuspended in hypotonic solution (1% sodium citrate in distilled water) for 10 min at room temperature. The cells were then fixed with ethyl alcohol-acetic acid (3:1). The fixative was changed three times and the cell pellet was resuspended in the fresh fixative each time. A drop of fixed-cell suspension was placed on a slide, permitted to dry, and was then stained with acetic orcein. A random sample of 40 to 50 well-spread metaphases was photographed for each subline, and chromosome counts were performed on projections of the developed films.

When accurate chromosome counts were not required, chromosome preparations were made by using the whole culture. After incubation with Colcemid, all subsequent treatments were carried out in the culture dish, taking care to minimize detachment of metaphase-arrested cells. Microscopic observations were performed directly on the cells that had been fixed, stained, and dried on the bottom of the culture dish.

Chemicals. 6-Thioguanine was obtained from Fluka A.G. Chemische Fabrik, Germany; aminopterin from Koch-Light Laboratories Ltd., England; 5-bromodeoxyuridine, thymidine, hypoxanthine, and glycine from Nutritional Biochemicals Corp., Cleveland, Ohio. Stock solutions were prepared in bidistilled water and kept frozen at −20 C.

Definition of ploidy. The terms "diploid" and "tetraploid" are used here to define cell lines displaying frequency distributions of chromosome numbers with a mode in the Syrian hamster diploid (44 ± 2) or tetraploid (88 ± 4) range. Cell lines with chromosome distributions showing modes higher than 44 ± 2, but not necessarily falling in the tetraploid range, are generically defined "polyploid."

Definition of morphology. A cell line is defined "morphologically normal" when the cells in monolayers show parallel orientation and no tendency towards multilayered growth and when, macroscopically, the monolayers display a regular "swirly" pattern. It is defined "morphologically transformed" when the cells in monolayers show no tendency towards parallel orientation, grow in a criss-cross, multilayered pattern, and macroscopically the monolayers appear either dense and uniform, or diffusely clumpy (Fig. 1).

RESULTS

Search for "revertants" in transformed tetraploid lines. The karyotype of line BHK21/13 is pseudodiploid, i.e., most cells have 44 chromosomes and a variable proportion have 88 or more (18). Although, by scoring metaphases, the proportion of polyploid sets may occasionally be as high as 10%, they do not all represent cells capable of giving rise to progeny, because the frequency of pure polyploid sublines obtained by cloning is lower than 10%. By repeated clonal isolation, however, a number of pure polyploid sublines with modes close to the tetraploid value were obtained. Most of these sublines still preserved a normal phenotype and could be transformed by polyoma virus at a rate similar to that of closely related diploid sublines (15).

Several transformed polyploid sublines were carried on for about 20 passages (120 generations). Soon after isolation and by the end of this period of culturing, the sublines were tested for the presence of "revertants" by plating several hundred cells and scoring for colonies of normal morphology. No such colony was ever observed. On the other hand, chromosome counts performed on a random sample of clones derived from the transformed polyploid sublines showed, after 20 passages, that the karyotype of these sublines had not changed significantly.

These experiments showed that, among polyploid cells transformed by polyoma virus, reversion, if it occurs, is a rare event and, therefore, would require a selective method to be detected. In the following paragraphs we describe the isolation of a polyploid line, obtained by hybridization of two variant BHK21/13 sublines, which was intended to provide the selective system required.

Isolation of variant BHK21/13 sublines. A subline of BHK21/13 which essentially lacks the enzyme thymidine kinase (B1) was isolated by virtue of its resistance to 5-bromodeoxyuridine (12). These cells are unable to phosphorylate 5-bromodeoxyuridine and, therefore, are protected against the lethal effect of incorporating this

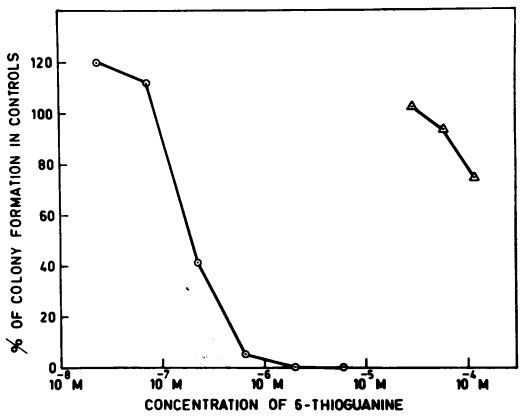

FIG. 1. *Sensitivities of BHK21/13* (○) *and the T6a subline* (△) *to 6-thioguanine. Two hundred cells in 60-mm petri dishes were exposed to medium containing 6-thioguanine in each concentration. The average number of colonies in two or three dishes for each concentration is expressed as the percentage of the number of colonies in similar dishes not containing the analogue. In these controls, the plating efficiency of BHK21/13 cells was 27%, and the plating efficiency of the T6a cells was 21%. The colonies of BHK21/13 which appeared in the presence of* 2.2×10^{-7} M *6-thioguanine, and especially of* 6.6×10^{-7} M *6-thioguanine, were small and toxic.*

nucleotide into DNA. In an analogous fashion, 8-azaguanine was used to select inosinic acid pyrophosphorylase-deficient sublines of mouse L cells (10). In BHK21/13 cells, Subak-Sharpe (19) found 6-thioguanine to be more toxic than 8-azaguanine; with 6-thioguanine, he isolated a subline of BHK21/13 cells with a markedly reduced ability to incorporate hypoxanthine (5% of controls), and, from transformed BHK21/13 cells, sublines essentially unable to incorporate hypoxanthine.

To obtain a subline of BHK21/13 essentially lacking inosinic acid pyrophosphorylase, we first determined the sensitivity of BHK21/13 to 6-thioguanine (Fig. 1). Then, 2×10^5 BHK21/13 cells in 100-mm petri dishes were exposed to medium containing 6.6×10^{-7} M 6-thioguanine. After 2 weeks of incubation, an average of 8.5 colonies per dish appeared. An extract of the cells from one such dish (T1) contained a reduced specific activity of inosinic acid pyrophosphorylase (Table 1). To obtain sublines essentially lacking the enzyme, these T1 cells (10^6 cells per 100-mm petri dish) were exposed to medium containing 4.2×10^{-5} M 6-thioguanine. An average of two colonies per dish appeared after 2 weeks of incubation. Several such colonies were isolated, and extracts of these sublines, T2, T3, T4, and T6, were subsequently shown to contain essentially no inosinic acid pyrophosphorylase activity (Table 1).

By exposing these sublines to medium containing 10^{-5} M aminopterin, 4×10^{-5} M thymidine, 10^{-4} M hypoxanthine, and 10^{-5} M glycine, it was possible to determine the frequency of cells which had regained the enzyme, as was done previously for B1 (12). For the present experiments, the subline (T6) containing the lowest frequency of revertants (6×10^{-6}) was chosen for hybridization with B1. T6 was also cloned to improve its plating efficiency, and the sensitivity of this subline (T6a) to 6-thioguanine was determined (Fig. 1); it was approximately 1,000 times more resistant to 6-thioguanine than was BHK21/13. Karyologically, B1 and T6 were both in the diploid range (15).

Isolation of hybrid cell lines. For hybridization, T6 and B1 cultures were treated with 3×10^{-5} M 6-thioguanine or 10^{-4} M 5-bromodeoxyuridine for several days to remove revertant cells. Then, approximately 5×10^5 cells of each subline, washed free of these analogues, were inoculated together in a 60-mm petri dish. In addition, the same number of cells from each subline, after removal of the analogue, were placed separately in five 100-mm petri dishes. The medium in all dishes contained hypoxanthine and thymidine to avoid the toxicity of any residual traces of 6-thioguanine or 5-bromodeoxyuridine. After 24 hr, 10^5 cells of the mixed culture were transferred to five petri dishes containing medium supplemented with aminopterin, thymidine, hypoxanthine, and glycine as described above; the dishes containing either B1 or T6 cells were also refed with this medium. After incubation for 8 days, there were no colonies in any of the

TABLE 1. *Pyrophosphorylase activities of BHK 21/13 sublines*

Subline	Activity	Percent
BHK21/13	1,495	100
T1	214	14.4
T2	0	0
T3	1.2	0.1
T4	0	0
T6	7.7	0.5

[a] Values represent μμmoles of phosphorylated derivatives of hypoxanthine-8-^{14}C synthesized in 1 hr at 37 C by the extract of 10^3 cells, assayed in duplicate and corrected for a blank reaction mixture lacking 5-phosphorylribose 1-pyrophosphate, as previously described (9). The value for BHK21/13 is the average value for three extracts. The subline T1 was selected for low resistance to 6-thioguanine, and from it were selected, for high resistance, the sublines T2, T3, T4, and T6, as described in the text.

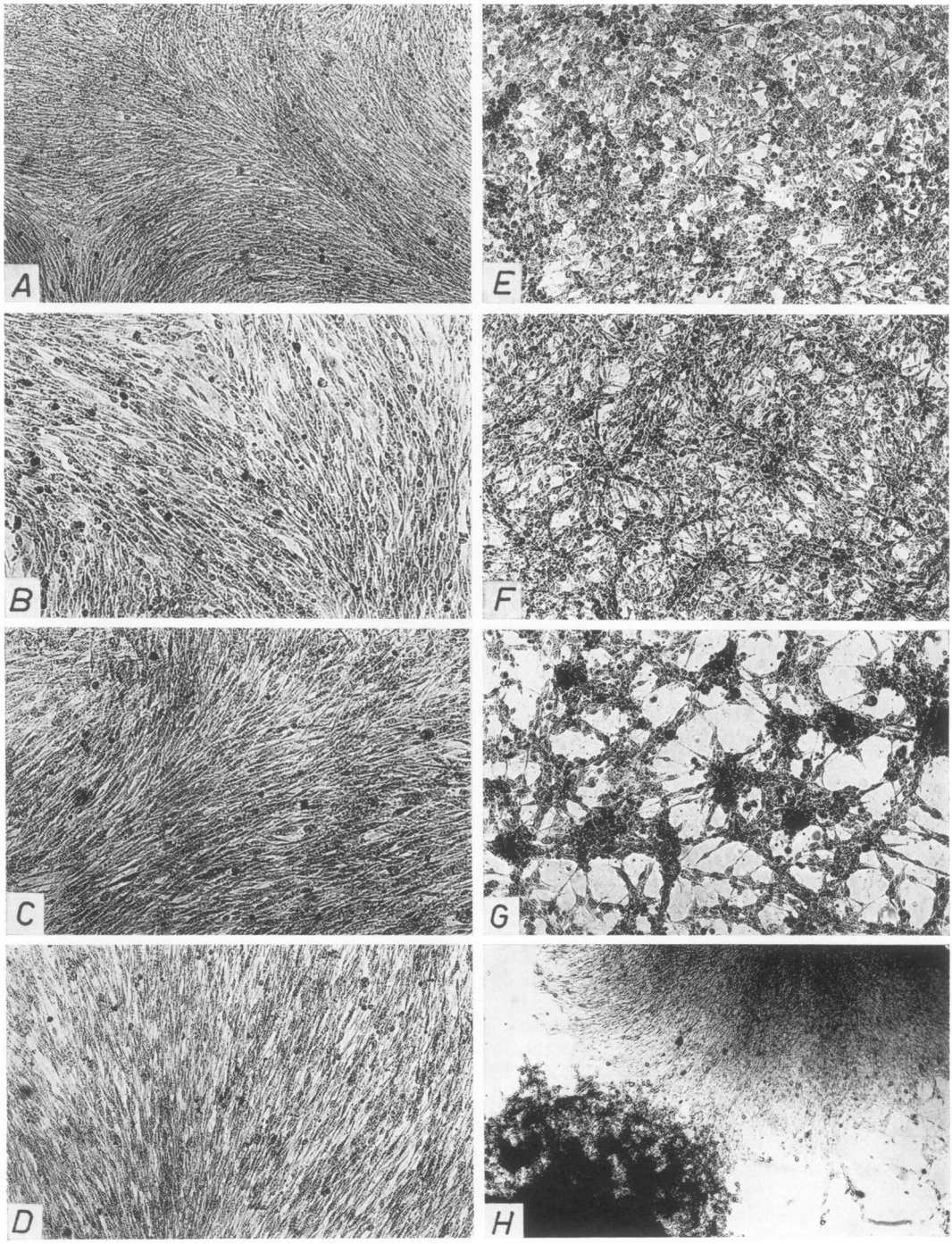

FIG. 2. *Morphology of line BHK21/13 and of various normal and transformed sublines. (A) Normal line BHK 21/13; (B) normal hybrid subline Hy3; (E) transformed subline Hy3-5; (C, D) normal (revertant) sublines derived from Hy3-5; (F, G), transformed sublines derived from Hy3-5; (H) a transformed and a normal colony grown from Hy3-5 survivors to 6-thioguanine. All photographs from A to G are at same magnification.*

TABLE 2. *Frequency distribution of chromosome numbers in different BHK21/13 sublines.*

Subline	No. of chromosomes																
	50					55					60					65	
Hy3....																	
Hy3-5...																	
ST1. ...			1			1	1		12	7	5	9	4	2	3		1
ST2....								1	2		2	5	6	3	3	19	1
ST4....	1	1	1	9	3	1	12	2	2	6	1	1	1				
ST5....		1	1		1	2	10	5		19	1	5	2		1		
ST2-6...			1				4		3	1	13		9	4	16		
ST2-10..							2	5	4	20	7	5	2	2	1	1	
ST2-25..	2				2	4	2	9	19	3	4		2	2	1		
ST2-28..									2	2	3	3	2	16	7	2	3
ST2-23..				1	1			2	7	8	13	4	10	1	4	2	
ST2-14..	1				1		1	1			2		10	5	18	2	1
ST2-3...		1									2	3	4	6	4	7	12
ST2-7...				1		1		10		6	4	1	12	1	12	1	
ST13...		6	11	7	5	12	4	7	4	4			1				
ST17...			2	1	1		3	13	8	5	7		1	2		1	1

Subline	No. of chromosomes (cont.)															
				70					75					80		
Hy3....					2		3	1	6	3	10	5	2	3	4	1
Hy3-5...				1		2	7	5	10	3	3	5		1		
ST1. ...																
ST2....	1															
ST4....																
ST5....																
ST2-6...		2														
ST2-10..																
ST2-25..																
ST2-28..	3	1														
ST2-23..																
ST2-14..	1	1														
ST2-3...	1	1														
ST2-7...																
ST13...																
ST17...																

[a] The characteristics of each subline are described in the text. Subline ST3 (not shown above) had a mode between 100 and 110.

TABLE 3. *Survival of BHK21/13 sublines to different drugs*[a]

Subline[b]	Drug tested					
	5-Bromodeoxyuridine (10^{-4} M)	Aminopterin[c] (10^{-5} M)	6-Thioguanine			
			2×10^{-5} M	10^{-5} M	5×10^{-6} M	2.5×10^{-6} M
Hy3ö..........	—	—	9	5	3	6
Hy3-5..........	0	Confluent	53	42	50	32
ST2............	—	—	Confluent	—	—	—
ST13...........	—	0	Confluent	—	—	—
ST17...........	—	43	Confluent	—	—	—
2P3-13..........	—	—	0	0	0	0
2P3-8...........	—	—	0	0	0	0
Tr4.............	—	—	0	0	0	0

[a] Cells were incubated in fluid growth medium containing the given concentration of drug until macroscopic colonies developed. Figures indicate the total number of colonies grown from 800,000 (eight plates, 100,000 cells per plate). "Confluent" indicates that, in the presence of the drug, 100,000 cells per plate (or less) readily grew to confluence, with no indication of cell death or reduced rate of multiplication.

[b] The characteristics of each subline are described in the text.

[c] Plus $1\theta^{-5}$ M glycine, 4×10^{-5} M thymidine, and 10^{-4} M hypoxanthine.

dishes containing B1 or T6 cells alone, but an average of 10 colonies of varying size in each dish from the mixed culture. When chromosomes were studied directly in one such dish, almost all of these colonies consisted of cells which were approximately tetraploid. Presumably, they represented hybrid cells resulting from the fusion of B1 and T6 cells. This same technique was used to study hybridization of mouse L cells (11); in other systems, it has been possible to show conclusively, with marker chromosomes, that fusion of two cell types has occurred (2).

Several hybrid colonies were isolated, and one (Hy3) was used in the present experiments.

Transformation of hybrid cells and selection for parental types. Hy3 cells were larger than normal BHK21/13 cells but displayed normal morphology (Fig. 2) and did not grow in soft agar. Upon infection with polyoma virus (400 plaque-forming units per cell), the absolute transformation frequency was 1 to 2% (15). A transformed colony (Hy3-5) was isolated, recloned, and tested for its sensitivity to aminopterin, 5-bromodeoxyuridine, and 6-thioguanine. The distribution of chro-

mosome numbers in this subline is shown in Table 2. Hy3-5 was still as fully resistant to 10^{-5} M aminopterin as Hy3, and had regained normal sensitivity to 5-bromodeoxyuridine and 6-thioguanine (Table 3). No survivors were obtained when 10^6 Hy3-5 cells were plated in the presence of 10^{-4} M 5-bromodeoxyuridine. In the presence of 6-thioguanine, survival was of the order of 10^{-4} for a rather wide range of concentrations of the drug (2×10^{-5} to 2.5×10^{-6} M), as shown in Table 3. This lack of dose-relationship observed with 6-thioguanine is suggestive of an "all-or-none" effect, and might be expected if resistance was due to the deletion of loci controlling the synthesis of inosinic acid pyrophosphorylase. It is possible that the hybrid cell had an unstable chromosome complement (8), and that occasional variants which had lost, among others, the chromosome or chromosomes concerned with the incorporation of purines, were able to grow in the presence of the analogue.

To check this possibility, the chromosome complement of a number of survivors was determined. As shown in Table 2, all the sublines tested, except one, had a distribution of chromosome numbers with a mean significantly lower than that of Hy3-5. Two of these sublines (ST13 and ST17) were tested for their sensitivity to 6-thioguanine and to aminopterin after about 4 weeks of culture (50 generations). Both were found to be still fully resistant to 2×10^{-5} M 6-thioguanine, and, as expected, were sensitive to 10^{-5} M aminopterin, although not quite as sensitive as the original B1 and T6 variants (Table 3).

Additional support for the hypothesis, that survival of Hy3-5 to 6-thioguanine was due to the selection of segregants of T6 parental type, was obtained by comparing the behavior of this subline to other nonhybrid transformed lines. Two polyploid-transformed sublines (2P3-8 and 2P3-13), obtained by infecting two different naturally occurring tetraploid BHK21/31 clonal derivatives and one diploid-transformed BHK21/13 subline (Tr4), were grown in the presence of various concentrations of 6-thioguanine. None of these sublines produced true survivors (i.e., colony-forming units) in the presence of 6-thioguanine at a concentration as low as 2.5×10^{-6} M (Table 3).

Occurrence of "revertants" among Hy3-5 survivors to 6-thioguanine. A number of sublines were obtained from a random sample of Hy3-5 survivors to 2×10^{-5} M 6-thioguanine. The morphology of these sublines was recorded (Fig. 2), and their ability to grow in soft agar was determined. In one experiment (Table 4, experiment 1), some of the sublines showed lower plating efficiency in soft agar medium than untreated Hy3-5 cells. Subline ST2, which had the lowest plating efficiency in agar, appeared also morphologically normal. Eight clones were derived from ST2 and were again tested for their ability to grow in soft agar. Five of these clones were morphologically normal and displayed low plating efficiency (approximately 20%); two were morphologically transformed and displayed higher plating efficiency in soft agar (63 and 88%); one showed intermediate morphology and plating efficiency (Table 4, experiment 2). The karyotype of all these clones was similar to that of the parental ST2 subline (Table 2).

In a different experiment (Table 4, experiment 3), a random sample of sublines derived from Hy3-5 survivors to 2×10^{-5} M 6-thioguanine were again tested for their ability to grow in soft agar, and were compared to a similar sample of clones derived from untreated Hy3-5 cells. In this case, the incidence of sublines displaying low plating efficiency in soft agar was higher than in the previous experiment. In Table 4, the morphology of these sublines is expressed as the approximate frequency of normal colonies observed by plating each subline separately, at a low cell concentration in liquid medium. It appears that sublines giving a high frequency of morphologically normal colonies are those with the least ability to grow in soft agar. Samples of clones derived from two typical sublines (ST13 and ST17) were shown to breed true with regard to morphology, as well as ability to grow in soft agar, if tested after several passages. The same behavior was displayed by clones of either morphological type isolated from one of the intermediate sublines (ST14).

DISCUSSION

The experiments described show that morphological transformation of hamster cells induced by polyoma virus is not irreversible. Furthermore, they point towards a direct chromosomal control of the transformed phenotype. It is extremely unlikely that the selection of contaminant untransformed cells might explain our results, for the following reasons. (i) The Hy3-5 line was obtained by recloning, in soft agar medium, a transformed colony collected from a soft agar plate of infected Hy3 cells. (ii) Survival of Hy3 cells to 6-thioguanine is nearly 10 times lower than that of Hy3-5 cells when the two lines are tested separately (Table 3). (iii) The distribution of chromosome numbers in Hy3-5 survivors to 6-thioguanine is quite distinct from that of Hy3 (Table 2).

Macpherson (13) has reported the reversion

TABLE 4. *Frequency of transformed and normal cells in sublines derived from Hy3-5 survivors to 6-thioguanine*

Expt no.	Subline	Morphology[a]	Plating[b] efficiency in fluid medium (*a*)	Plating[c] efficiency in agar medium (*b*)	Plating efficiency in agar medium of viable cells ($b/a \times 100$)
			%	%	%
1	Hy3	Normal	33.4	0.1	0.3
	Hy3-5	Transformed	40.6	39.3	96.7
	ST1[d]	Transformed	17.7	9.8	55.5
	ST2	"Normal"	38.4	9.3	24.3
	ST3	Transformed	30.7	15.3	49.3
	ST4	Transformed	18.7	8.5	45.3
	ST5	Transformed	29.6	20.4	68.8
	ST6	Transformed	21.6	13.1	60.8
2	ST2-6[e]	Intermediate	25.0	12.2	48.7
	ST2-10	Transformed	26.4	16.6	62.9
	ST2-25	Transformed	25.1	22.1	88.0
	ST2-28	Normal	40.2	7.8	19.3
	ST2-23	Normal	46.7	8.2	17.6
	ST2-14	Normal	53.1	11.2	21.1
	ST2-3	Normal	40.6	10.3	25.4
	ST2-7	Normal	28.4	6.8	23.9
3	Hy3-5-1[f]	0	33.2	33.4	100.5
	Hy3-5-2	0	52.6	47.8	90.8
	Hy3-5-3	0	68.5	61.6	89.9
	Hy3-5-4	0	46.2	42.1	91.2
	Hy3-5-5	0	44.1	46.0	104.4
	Hy3-5-6	0	35.4	34.5	97.4
	Hy3-5-7	0	47.2	45.9	97.3
	Hy3-5-8	0	31.1	32.7	105.1
	Hy3-5-9	0	21.0	21.5	102.4
	Hy3-5-10	0	27.7	27.6	99.8
	ST12[d]	0	10.2	8.7	85.5
	ST13	0	32.0	28.3	88.5
	ST15	0	32.5	28.9	89.0
	ST16	0	9.6	6.8	70.4
	ST20	10	18.7	2.1	11.3
	ST22	20	26.6	4.2	15.8
	ST19	20	33.6	5.9	17.4
	ST11	30	9.7	1.4	14.2
	ST18	30	24.8	2.0	8.2
	ST14	50	31.6	7.1	22.4
	ST21	80	31.0	1.5	4.9
	ST17	>95	27.3	0.14	0.5

[a] In experiments 1 and 2, morphology was evaluated subjectively on confluent cell monolayers. In experiment 3, morphology was evaluated on single-cell platings and, therefore, is expressed as the approximate percentage of normal colonies.

[b] 200 Cells were plated per dish; 4 to 8 dishes were scored for colony counts.

[c] 1,000 Cells were plated per dish; 4 to 8 dishes were scored for colony counts.

[d] Sublines derived from Hy3-5 survivors to 2×10^{-5} M 6-thioguanine.

[e] Clonal sublines of ST2, from experiment 1.

[f] Clonal sublines of untreated Hy3-5.

of morphological transformation of BHK21/13 cells transformed by Rous sarcoma virus (Schmidt-Ruppin strain). However, the virus-cell interaction leading to transformation is presumably different in this system, where the viral genetic material is RNA. Since vegetative virus, as a rule, cannot be recovered in polyoma-transformed cells (7), operationally polyoma-induced transformation may be considered a true mutation, where a permanent modification

of the genome is transmitted to the progeny with no need for the mutagenic agent to be continuously present. Somatic cell hybridization experiments have indicated that such modification is not a deletion (4); on the contrary, the presence of RNA complementary to polyoma DNA in transformed cells suggests the permanent addition of viral genetic material (1).

The association of chromosome loss with the loss of the transformed phenotype strengthens the hypothesis that specific genes are directly responsible for the maintenance of the transformed characteristics. The genes involved could be either viral or cellular, or both. It should be noted that only one subline with fully "reverted" phenotype was obtained among direct Hy3-5 survivors to 6-thioguanine, i.e., subline ST17 (Table 4); most of the other selected sublines showed intermediate characteristics. Instability and segregation of normal and transformed cell types within each subline cannot entirely account for this result, since, in at least one case, the cloning of a morphologically "normal" subline which showed decreased plating efficiency in soft agar did not yield pure "revertants" (Table 4, experiment 2). This quantitative aspect suggests a polygenic determination of the transformed phenotype. It is possible that the full expression of the transformed phenotype depends on the interplay of conditioning cellular genes and structural, possibly viral, genes. The loss of either type of genes could cause partial or full reversion to the normal phenotype. Our present data do not allow us to assess whether structural or conditioning genes might be lost in the cases observed.

If a direct correlation between loss of chromosomes and loss of the transformed phenotype could be confirmed, the selective system described here could be used to determine whether a specific chromosome (or group of chromosomes) is involved in transformation. Somatic cell hybridization has been considered a potential method for carrying out genetic analysis of somatic mammalian cells (8), mainly because hybrids prove to be karyotypically unstable and, thus, are a potential source of segregants (recombinants) (16). In the work reported here, the use of a specific biochemical marker provided a selective means of isolating such segregants. It might be that sublines with low chromosome numbers and recombinant phenotypes will eventually appear at a higher frequency in untreated Hy3-5 populations as a result of spontaneous progression of this line. If a sufficient number of Hy3-5 segregants could be tested for the presence of other characteristics induced by polyoma infection (transplantability in vivo, specific transplantation or complement-fixing antigens, RNA complementary to polyoma DNA, etc.), it would be possible to approach on a more quantitative basis the problems raised by the apparent lack of correlation among some of these characteristics (5).

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LITERATURE CITED

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