Steps in the Neoplastic Transformation of Hamster Embryo Cells by Polyoma Virus

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*STEPS IN THE NEOPLASTIC TRANSFORMATION OF HAMSTER EMBRYO CELLS BY POLYOMA VIRUS**

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Cultures of hamster embryo cells infected with polyoma virus undergo a characteristic transformation within several weeks after infection. The transformed cultures are constituted by highly atypical cells which have an abnormal morphology, grow rapidly *in vitro*, and give rise to progressively growing tumors when inoculated subcutaneously into the adult hamster.[1]

In the previous experiments, the transformed cells arose in mass cultures and their properties were only studied many cell generations after the original virus-cell interaction had taken place. The experiments to be reported in this communication were undertaken to get some information on the properties of transformed cells at earlier stages after infection with polyoma virus. The experiments show that the atypical cells are produced in two main steps; moreover, they reveal some new characteristics of the transformed cells which may contribute to a clarification of the mechanism of transformation.

Material and Methods.—The polyoma virus used was of the "large-plaque" type. Its origin and the preparation of its stocks have been described.[2] Tissue cultures of whole embryos of Golden hamsters were prepared according to a technique already described.[3]

A rich growth medium especially favorable for the cloning of mouse embryo cells (Weisberg, R., personal communication) and for the titration of polyoma-induced foci (Bayreuther, K., personal communication) was used for all experiments. It consisted of reinforced[4] Eagle's medium (40 pts), Puck's[5] nutrient solution N16 (40 pts), and medium NCTC 109 (4 pts); all ingredients were dissolved in Earle's saline containing 0.55 per cent glucose and 0.37 per cent $NaHCO_3$. This medium was further supplemented with 0.3 per cent Bacto tryptose phosphate broth (10 pts) and, unless stated differently, with 10 pts of fetal bovine serum.

Experimental procedure: Sets of secondary hamster embryo cultures, seeded with 8×10^5 cells per plate, were infected with polyoma virus: an aliquot of 0.1 ml of a virus suspension of a titer of 10^9 PFU/ml was distributed over the cells after removal of the fluid medium. Adsorption of the virus was allowed to proceed for 2 hr at 37°C in an incubator flushed with an adequate CO_2-air mixture; during this time, about 10 per cent of the input virus was adsorbed to the cells, as was shown by control experiments with purified, P^{32}-labeled, virus (unpublished). The cultures were washed three times with medium and overlaid with 5 ml of medium. The cultures were maintained under optimal growth conditions by three weekly fluid changes; the cells were transferred when the cell sheets became confluent.

At various times (2, 4, 9, 13, 16, and 24 days) after infection, single cultures of the sets were trypsinized and used to start a group of *sparse* subcultures. These cultures were seeded with 10^5 cells and grown for seven days in the presence of 20 per cent fetal bovine serum; thereafter, fluid changes were done with regular medium. The sparse subcultures were scanned on days 10 to 16 with a low-power inverted microscope for areas of high mitotic activity and/or abnormal morphology. At this time, the subcultures were mostly constituted by a confluent monolayer of cells showing almost no mitotic activity. Some cultures contained one or a few foci of cells of unusual morphology which grew in several layers and showed frequent mitoses. The cells of these foci were usually markedly refringent and fusiform in shape; they crisscrossed each other and sometimes piled up to form nodules or strands.

The number of foci obtained on sparse subcultures started within the first week after infection was about 10^{-5} of the cells plated; since the multiplicity of the adsorbed virus was 10–20, the efficiency of focus formation was about 10^{-6} that of plaque formation on mouse embryo cells.

A number of foci were picked from the sparse subcultures. To do so and to carry over the least amount of untransformed cells, small glass cylinders were used to isolate the foci from the surrounding cells.[6] The glass wells were filled with trypsin and the dispersed cells transferred to new petri dishes. In certain cases, the foci were aspirated with a capillary pipette to avoid the use of trypsin and transferred to petri dishes containing coverslips. The cell lines started from individual foci will be designated as *focal lines*. All focal lines derived from sparse subcultures started within the first two weeks after infection were isolated from cultures containing each a single focus. They can therefore be assumed to derive from a single transformed cell; however, the presence, at the outset, of a small proportion of contaminant nontransformed cells cannot be excluded.

The focal lines were maintained by a regular regime of fluid changes and transfers; where trypsin was to be avoided, the old coverslips were replaced at regular intervals by fresh coverslips which became repopulated by cells derived from the outgrowth surrounding the coverslips. Without exception, all focal lines could be maintained indefinitely in an actively growing state. The cells were characterized by their refringence, their high mitotic activity, and their capacity to form multilayered cell sheets with a crisscross pattern in dense cultures.

Two types of controls to the focal lines were done. One type consisted of lines started with cells from areas of the sparse subcultures outside of a focus = *nonfocal lines*. Usually, the cells from areas delimited by three glass cylinders were compounded to obtain enough cells to start each nonfocal line. These lines were constituted by cells with the characteristics of normal cells; they could not be maintained for more than a few passages.

The other type of control consisted in a repetition of the entire experimental procedure, as outlined, with the exception that the original sets of cultures were not infected with polyoma virus. In these control sets, no foci comparable to those of the infected sets were found in the sparse subcultures. Only a few areas of persistent mitotic activity but of normal morphology were seen. These areas were picked and *control lines* started. These lines could be maintained for only a few transfers. One exceptional line could be cultured for five transfers. This line consisted of very flat, transparent cells. It had other peculiar features to which we shall return.

Results.—The present investigation consisted in a study of the properties of the cells of the *focal* lines at various times after their isolation. The earliest observations were carried out at the second or third transfers when a sufficient number of cells were available. At this time, nontransformed cells which might have been picked with the focus when the line was started would have been considerably diluted

out owing to their limited growth ability, as demonstrated by the poor growth of control lines. The cells of the focal lines were characterized on the basis of the following properties: morphological types of the clones formed, efficiency of cloning, and transplantability into 20-day-old hamsters. The efficiency of cloning was measured by determining the proportion of cells capable of forming a clone of 200 or more cells after seven days of incubation in medium supplemented with 20 per cent fetal bovine serum (without feeder layer). The transplantability was determined by inoculating 5×10^6 cells from a trypsinized cell suspension subcutaneously into 20-day-old hamsters. The degree of transplantability was measured by the time required to form a tumor of the size of a walnut at the site of inoculation. The results of these determinations are summarized in Table 1. The following points should be emphasized.

The clones formed by the cells of the focal lines could be divided into two main morphological groups. One group consisted of "thin" clones in which the cells grew mainly in a single layer; at the early transfers, the thin clones had a very loose structure and were small in size (*ca.* 200 cells per clone); at later transfers, they became larger in size (1,000 and more cells) and showed a uniform crisscross-like pattern. All thin clones were clearly different from normal clones: the cells were refringent and randomly oriented. The thin clones were prevalent at the early transfers; their proportion decreased in later transfers. The second group of clones were "dense" clones, in which the cells piled up in many layers. The morphology of these dense clones was variable: some showed a uniform dense center, others had heavy strands, others nodules. These clones were absent or rare in early transfers and increased in proportion in later transfers.

Clones produced by cells from the nonfocal or control lines were of a strict monolayer type; the cells were thin and transparent and showed a regular arrangement. They were readily distinguishable from the thin clones just described. We can therefore conclude that both groups of clones formed by cells from focal lines were clones of transformed cells.

The efficiency of cloning of the cells of the focal lines at the early transfers was very similar to that of normal cells. At later transfers, this efficiency increased in parallel with the appearance of thin clones of large size and of dense clones.

The transplantability of the cells of the focal lines varied markedly from line to line, and within a given line, with the transfer number. In general, the transplantability was very low or absent at early transfers and increased with later transfers. The increase in transplantability proceeded at equal pace with the increase in the proportion of dense clones. These results suggested therefore that the highly transplantable cells were the same cells that gave rise to dense clones. This deduction was confirmed by comparing the transplantability of subcultures derived from eight clones of the thin type, obtained at later transfers when the clones formed were rather large, with that of subcultures of five dense clones. As shown in Table 2, the average time for developing a tumor of walnut size was 8.4 weeks for the cultures of the thin clones (2/35 animals did not develop any tumor) and 4.3 weeks for the cultures of dense clones (23/23 animals developed a tumor).

As to the relationship of the two types of cells present in the focal lines, two possibilities can be visualized: either the cells giving rise to dense clones are variants of the main cell type which forms thin clones or the two cell types originated in in-

TABLE 1

EFFICIENCY OF CLONING AND TRANSPLANTABILITY OF VARIOUS FOCAL LINES

Days after infection*	Focal line	Transfer	Efficiency of Cloning (%)†		Tumor of Walnut Size Within Weeks													No tumor ‡	Total number of animals§
			All clones	Dense clones	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
2	26	tr9,10	4.0(277)**	0.2				1		3									4
	43	tr3,4	0.2(106)	0.0															2(12) 2
		tr12	3.5(278)	0.2									1		1				2
	61	tr9–11	13.0(104)	1.0			1	1	1	2									5
4	79	tr4	0.5(69)	0.0								2							2
		tr6,7	1.3(51)	0.03						3	1								4
		tr10					2												2
	80	tr3								1		1							1(9) 3
		tr7–10	1.4(115)	0.4			3	4											7
9	25	tr3				3													3
	27	tr3							1	1		1							3
	28	tr2,3	2.5(97)	0.4															3(12) 3
	29	tr3								1			1	1					3
		tr7–10	17.9(143)	4.1			4	1	1										6
	78	tr3–6							2	6									8
		tr7–8							4	1	1								6
13	30	tr2										1	1	1					3
		tr5–9	2.1(168)	0.2								2	1				1		4
16	18	tr2					1	3											4
		tr4–6				5													5
		tr7–9	6.6(397)	2.2	5														5
	42	tr2													1				3
		tr6	1.9(151)	0.1								1							1
	45	tr2-4																	6(12) 6
		tr5-7																	5(13) 5
		tr8–9	9.0(74)	0.3									1		1	1		1	4
24	1	tr2,3	9.0(37)	0.0														1	1(13) 2
	2	tr9				3													3
	4	tr2,3	14.0(57)	2.0				2	1		1	1							5
		tr9					3												3
	5	tr10						3											3

* Time of seeding of sparse cultures.
† Proportion of clones formed over all cells plated.
‡ Numbers in parentheses indicate number of weeks of observation.
§ Twenty-day-old hamsters were inoculated with 5×10^6 to 10^7 cells of the focal lines indicated in column 2.
** The numbers in parentheses indicate the total number of clones counted.

TABLE 2

TRANSPLANTABILITY OF CLONAL DERIVATIVES OF FOCAL LINE 79

Type of clone	Clone	Transfer	Tumor of Walnut Size Within Weeks 4	5	6	7	8	9	10	11	12	13	No tumor	Total number of animals
Thin	5	4											2(12)	2
	8	6			3	1	1							5
	11	2						3		1				4
	14	4, 7				1	2					1		4
	15*	3, 7			1	1	1	2						5
	15-A2	3			1	1	3							5
	15-C1	2		1		3		1						5
	15-C2	2								1	4			5
Total				1	5	7	7	6		2	4	1	2	35
Dense	13	2	1	3										4
	15-A7	3	5											5
	15-A10	3	5											5
	15-A12	3	3	2										5
	15-C3	2	3											3
Total			17	5										22

* Clone derived from a single-cell isolation.

dependent events and were accidentally mixed in the focus. Two lines of experimental evidence point to the first possibility.

The first type of evidence derives from the study of the properties of cells of nonfocal lines. If cells with the capacity to form dense clones had been scattered all over the sparse cultures but inhibited by the surrounding cells from forming a focus, cell platings from the nonfocal lines should have given rise to dense clones. This was never observed.

The second line of evidence comes from the study of a clonal line derived from a single cell of a focal culture. The single-cell isolation, which was done by C. Basilico, was performed under microscopic observation and by using X-rayed mouse embryo cells as feeder-layer. The cell was isolated when the focal line was at its eighth transfer, i.e., 56 days after the original infection. Many attempts to isolate single cells from the first or second transfer of focal lines had been unsuccessful for unforseeable difficulties (see later). At the early transfers, the clonal line gave rise to clones of the thin type only. After six weeks of culturing, however, dense clones of the nodular, strand, and uniform type appeared in a low proportion (0.01 per cent). Cultures from these dense clones gave rise to tumors of walnut size within four weeks (Table 2). Cells forming clones of the dense type had a strong selective advantage *in vitro* and tended to outgrow the other type. This explains why in previous experiments cultures of hamster embryo cells infected with polyoma virus and maintained as mass cultures by regular transfers became ultimately converted into pure cultures of cells of the dense clone type.[7]

The cells of the focal lines were examined for two other properties: the presence of infectious polyoma virus and the presence of chromatid breaks.

The absence of demonstrable virus in the mass cultures of transformed hamster embryo cells at later stages has already been reported, as well as the impossibility of inducing virus release from these cells.[2] Similar negative results were obtained with the focal cultures at early transfers. At the second transfers, 10 to 20 plaque-forming units of polyoma virus were usually found per culture; at the third and later transfers, the cultures contained no infectious virus. Induction experiments

were carried out at the third to sixth transfer of the focal lines. No production of infectious virus or of infectious viral DNA could be obtained after thymidine starvation induced by aminopterin as previously described. X-rays and over-crowding were equally ineffective as inducing agents.

The search for chromatid breaks was prompted by the presence of an often unusually high number of dead cells in the cultures of the focal lines at a time when no polyoma virus could be detected. A measure for chromatid breakage was obtained by scoring mitotic figures at late anaphase and early telophase for the presence of chromatid bridges. Coverslip cultures were prepared at various transfers

TABLE 3

FREQUENCY OF CHROMATID BRIDGES IN FOCAL, NONFOCAL, AND CONTROL LINES

Type of cell line	Line	Transfer	Number of bridges per anaphase 0	1	2	3	4	>4	n	Percentage of anaphases with bridges
Focal	592-B1	tr2 (27)‡	23	11	16	18	17	15	100	77.0
		tr3 (31)	16	16	8	4	4	2	50	68.0
	592-B2	tr3 (31)	19	9	3	4	6	9	50	62.0
		tr6 (43)	14	13	10	2	5	6	50	72.0
	612-F41	tr3 (37)	47	25	11	4	7	6	100	53.0
	614-E30	nt* (35)	8	12	7	12	6	5	50	84.0
	614-E98	nt (35)	39	24	17	10	4	6	100	61.0
	620-L48	tr1 (17)	40	15	14	15	4	12	100	60.0
		nt (26)	40	16	15	9	9	11	100	60.0
	622-M26	nt (21)	15	6	6	8	3	2	40	62.5
Non-focal	592-NoF4	tr2 (31)	185	8	3	1	2	1	200	7.5
	612-NoF2	tr1 (23)	88	5	6	1			100	12 0
	612-NoF5	tr1 (23)	97	2			1		100	3.0
	612-NoF9	tr1 (23)	80	12	6	1		1	100	20.0
	612-NoF22	tr1 (24)	91	4	3	1		1	100	9.0
	614-NoF22	tr2 (21)	95	3	1			1	100	5.0
		tr3 (25)	91	2	3	1	1	2	100	9.0
		tr4 (29)	90	6	3	1			100	10.0
Control	592-CoB5	tr2 (24)	91	6		2		1	100	9.0
		tr4 (31)	166	8	7	6	4	9	200	17.0
	598-CoD1	tr2 (25)	91	2	2	1	2	2	100	9.0
	598-CoD2	tr2 (25)	93	3	1	2		1	100	7.0
	598-CoD3	tr2 (25)	90	5	3		2		100	10.0
	588-CoC1-1†	tr4 (28)	34	7	6		1		48	29.0

* nt = no trypsin was used for the growth of these lines.
† This line was derived from an area of unusually high activity.
‡ Numbers in brackets = days after original infection.

of the focal lines; the coverslips were stained with acetic-orcein after fixation with acetic acid-alcohol (3:2).

The results of this investigation are given in Tables 3 and 4. They showed that the proportion of dead cells, as estimated semi-quantitatively, correlated well with the proportion of mitoses which contained chromatid bridges. This proportion was without exception strikingly high (53–84 per cent) at the early transfers; at later transfers, it decreased and progressively tended to that of the controls. The decrease occurred at a variable rate in different focal lines. In general, an abnormal occurrence of bridges could be observed for at least six transfers.

To evaluate the relationship of the presence of chromatid breaks with the transformation of the cells by polyoma virus, the frequency of chromatid bridges in

nonfocal lines and in noninfected control lines is also entered in Table 3. As can be seen from the table, both types of control lines showed a significantly smaller proportion of mitoses with bridges than the focal lines. In these controls, the highest frequency of bridges (29 per cent) was found in a clone of normal cells already mentioned, which showed an unusually high growth ability. Whether these two properties are correlated is unknown.

Since trypsinization is known to induce chromatid breakage,[8] some of the focal lines were grown without trypsinization. The results showed no difference between trypsinized and nontrypsinized cultures.

These observations leave therefore no doubt that the production of chromatid breaks leading to bridge formation is characteristic of the transformation induced by polyoma virus in this *in vitro* system.

TABLE 4

FREQUENCY OF CHROMATID BRIDGES AFTER LONGER PERIODS OF CULTIVATION

Focal line	Transfer	Number of bridges per anaphase							Percentage of anaphases with bridges
		0	1	2	3	4	>4	*n*	
592-B1	tr2 (27)†	23	11	16	18	17	15	100	77.0
	tr3 (31)	16	16	8	4	4	2	50	68.0
	tr6 (43)	23	7	6	7	2	5	50	54.0
	tr10 (56)	71	17	3	4	3	2	100	29.0
592-B1-F1*	tr9 (62)	64	27	4	4	1		100	36.0
592-B1-F2*	tr9 (62)	63	19	12	4	2		100	37.0
592-B1-F3*	tr9 (62)	80	10	5	5			100	20.0
568-26	tr6 (60)	34	12	9	2	3	4	64	47.0
568-43	tr9 (68)	48	17	9	3	7	9	93	48.0
568-61	tr7 (63)	98	43	11	8	2	9	171	43.0
570-80	tr6 (61)	33	6	4	3	1	1	48	31.0
	tr8 (73)	55	4					59	7.0
572-28-6	tr6 (75)	27	17	9	4	1	8	66	59.0
572-29	tr9 (60)	82	12		3		3	100	18.0
	tr12 (74)	79	13	2	2		4	100	21.0
572-78	tr5 (61)	95	4	1				100	5.0
574-30	tr8 (69)	65	20	6	1	1	7	100	35.0
576-18	tr6 (62)	71	21	3	3	1	1	100	29.0
	tr9 (75)	87	9	1	1	1	1	100	13.0
	tr11 (78)	81	17	1			1	100	19.0
580-4	tr9 (84)	88	10				2	100	12.0

* Subcultures from dense clones.
† Numbers in brackets = days after original infection.

Discussion.—The focal lines derived from the infected cultures contain various cell types of which none is present in noninfected cultures. The cell types can be grouped into two main classes: cells forming thin clones and cells forming dense clones. The results described above lead to the conclusion that cells forming thin clones are produced by the primary action of the virus, whereas cells forming dense clones arise from the former type by variation. Only cells forming dense clones have a high degree of transplantability. The neoplastic transformation of normal hamster embryo cells caused by polyoma virus occurs therefore in at least two steps and the action of the virus is twofold: on one hand, it causes the primary transformation of the cells; on the other hand, it provides the mechanism for the additional variation of the transformed cells.

By using a tissue-culture-adapted, rapidly growing line of hamster cells, MacPher-

son and Stoker[10] have obtained transformation to the malignant state in a single step. This finding need not be at variance with the results of the present investigation. In fact, the cells of the line used by these investigators have already spontaneously undergone profound changes which allow their unlimited and rapid multiplication *in vitro*. Thus, a single additional step, caused by the virus, may be sufficient to transform these cells to a malignant state.

The evolution of the focal cultures described in this article is probably one aspect of the phenomenon of *progression*[9] which has been recognized as a constant property of cancers in animals. In the present experiments, the progression appears to be, at least to a certain extent, a necessary consequence of the primary transformation and not a superimposed accidental complication.

Chromatid breaks were observed in a large proportion of the cells of most focal cultures, with the highest frequency during the early transfers. This finding raises the question whether the progression observed in this system is due to gross chromosomal alterations caused by the breaks; such a view would be supported by the preliminary findings of aneuploidy in cells which form dense clones. On the other hand, the chromatid breaks may not be directly involved in the progression; they could rather be a symptom of irregularities in the process of DNA replication in the transformed cells, able to cause several different consequences, including the progression. The inability to find chromatid breaks in one focal line (572–78) may be attributed to a precocious healing of the process causing the breaks, which tends to heal in all lines at later transfers.

The present observations are relevant to the problem of the fate of the viral DNA in the transformed cells. The results previously obtained, that the cells of the dense clones do not contain the viral nucleic acid in a state detectable by infectivity, has now been extended; it has in fact been shown that this is true also for the cells of the thin clones. The results still leave the question open whether the virus-cell interaction which leads to transformation is nonproductive from the start or becomes so after a small number of cell generations.

As pointed out in the past,[2] these results show that autonomously replicating polyoma virus DNA is not present in the transformed cells; they do not, however, exclude the presence of an integrated viral DNA. A new point in favor of the latter possibility is the production of chromatid breaks in the focal lines. These breaks, which are characteristic of the transformed hamster embryo cells, continue to be produced after infectious virus has disappearaed. A continued production of chromosomal alterations and of chromatid breaks has also been observed in human cells transformed by the simian SV40 virus,[11, 12] which is related to polyoma virus, and in cell lines of the Chinese hamster which survived an infection with the DNA-containing herpes virus.[13, 14] The continued production of chromosomal alterations suggests that a specific agent—possibly the viral DNA—is continuously present in these cells after the infection.

Functionally, the primary action of the virus in the transformation of the hamster embryo cells appears to be a release of the regulation of cell multiplication; this is revealed by the ability of the cells of the thin-clone type to form foci. In cells in which this original change has taken place, some of the chromosomal aberrations subsequently produced acquire, at least *in vitro*, a selective value. A stepwise process in this system leads thus finally to the malignant cell.

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*SPIN $^1/_2$ WAVE EQUATION IN DE-SITTER SPACE**

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1. *Introduction.*—The de-Sitter space can be represented by a 4-dimensional pseudo-sphere embedded in a 5-dimensional space. Let $\xi_\mu (\mu = 1, 2, \ldots, 5)$ be the five real coordinates of the 5-dimensional space. The de-Sitter space is described by the surface

$$\xi^\mu \xi_\mu = R^2, \tag{1}$$

where

$$\xi^\mu = \eta^{\mu\nu} \xi_\nu. \tag{2}$$

$\eta^{\mu\nu}$ is the (μ, ν)th matrix element of the diagonal matrix

$$\eta = \begin{pmatrix} 1 & & & & \\ & 1 & & & \\ & & 1 & & \\ & & & -1 & \\ & & & & 1 \end{pmatrix}, \tag{3}$$

and the real constant R is the radius of the pseudo-sphere. The totality of all homogeneous linear transformations among ξ^μ that leave the surface (1) invariant forms a ten-parameter group. In the limit $R \to \infty$, the de-Sitter space becomes simply the usual flat 4-dimensional space, and this ten-parameter group reduces to that of the inhomogeneous Lorentz transformation.