*RAPID TRANSFORMATION OF HUMAN FIBROBLAST CULTURES BY SIMIAN VIRUS 40**

By F. Jensen, H. Koprowski, and J. A. Pontén

THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY, PHILADELPHIA

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Cultures of normal human fibroblasts are characterized by a finite lifetime *in vitro*.[1] Actively proliferating cells can usually be maintained in culture for only 35–40 transfers. Following this period the growth rate of the cultures gradually declines, and proliferation ceases completely after 5–10 further passages.[1, 2]

In previously reported studies[3–6] on transformation of human cell cultures by simian virus 40 (SV40), either primary explants or actively proliferating established cell strains were exposed to SV40. In this study the susceptibility to SV40 of cultures approaching the end of their expected *in vitro* lifetime was assessed. It was found that these aging cultures transformed considerably earlier after infection than the primary explants and "young" actively proliferating cell strains examined before.

Materials and Methods.—The WI-26 and WI-38 human fibroblast strains were originally obtained by cultivation of embryonic lung tissue.[1] Cells of two WI-26 substrains used in the present study (Nos. XII and XXVI, Table 1) could not be transferred beyond the 41st and 56th subcultures, respectively. Each of three parallel cultures of substrain XXVI was exposed to SV40 at the 55th subculture. Cells of the parent WI-38 strain and of its No. IV substrain did not survive subculturing beyond the 50th and 48th passages, respectively. As shown in Table 1, these cultures were exposed to SV40 at the level of the 50th and 47th subcultures.

The W-5 CM strain originated from an explant of a fragment of buccal mucosa obtained from a 72-year-old man who had been treated with X rays and cytoxan after surgical removal of a bronchial carcinoma.[3] Following formation of a monolayer, the cell strain was propagated in actively proliferating cultures up to the 23rd transfer, when the cultures were frozen and stored, but could not be recovered in a viable state after thawing.

Two substrains (D 90 and D 285) were established from the 12th passage of the parental W-5 CM strain after the culture flasks were left in an incubator for 90 and 285 days, respectively, without a change of nutrient medium. Although this prolonged incubation period led to a cessation of mitosis and some cell loss, the remaining cells of the two cultures started to proliferate again when fresh medium was supplied. However, cells of substrain D 90 could be subcultured only at weekly intervals as compared to biweekly transfers for the parent strain. The cell growth of D 90 slowed down even more at the level of the 19th passage, and the cultures could not survive beyond the 25th–30th passage (Table 1). Each of the three parallel cultures of this substrain was exposed to SV40 at the 21st, 21st, and 22nd passages, respectively (Table 1).

Three parallel cultures of substrain W-5 D 285 (Table 1) were infected with SV40 at the 13th passage, i.e., the first passage following restoration of cultures after prolonged incubation. Although parallel noninfected cultures of this strain are still maintained, growth is so slow that they can be subcultured only at intervals of 2–3 weeks.

TABLE 1

TRANSFORMATION OF HUMAN FIBROBLASTS EXPOSED TO SV40 AT DIFFERENT PASSAGE LEVELS *in vitro*

Characteristics of the Cultures				
Strain	Substrain	Maximum number of passages obtained with noninfected parallel cultures	Passage at time of exposure	Time interval between exposure and morphologic transformation (weeks)
WI-26	XII	41	27*	10
	XXVI	56	55	5
		56	55	5
		56	55	3
WI-38	Parental	50	9*	8
			25*	8
			50	4½
	IV	48	47	4½
W-5 CM	Parental	>23†	Primary explant‡	9
	D 90	25	21	4
		27	21	3
		30	22‡	2½
	D 285		13	3
			13	3½
			13‡	4

* Observation previously reported.[6]
† See text.
‡ Green monkey kidney pool of SV40 used for infection; in all other cases human tissue pool of virus was used.

Two pools of SV40 virus were used for infection of tissue cultures—one prepared in green monkey kidney, and the other obtained from chronically infected transformed cultures of human cells.[6] General handling of normal and infected cultures and techniques used for morphological examinations did not differ from those reported before.[3, 6] Chromosome analysis was performed on cell samples obtained from cultures at different passage levels before and after exposure to SV40. The chromosome preparation technique used in this study has been described in detail elsewhere.[2, 7]

Results.—Results shown in the 3rd and 4th columns of Table 1 indicate that many of the cell strains were indeed at the end of their *in vitro* lifetime at the time of their exposure to SV40. Their rate of growth was very slow, and there was virtual absence of mitotic figures. However, within two weeks after infection, the appearance of the cultures began to change. Mitotic figures and foci with increased cell density were clearly visible, and a slight cytopathic effect was noted. None of these changes was observed in noninfected control cultures.

Morphological transformation occurred within two weeks after the first increase in mitotic rate was observed. Definite transformation was considered to have taken place when the following three criteria were met: (1) "loss of mitotic inhibition," i.e., presence of numerous mitotic figures even in areas of confluent cells; (2) disorganized multilayer growth; and (3) occurrence of epithelial-like cells. This corresponds to "early transformation," as defined previously.[6] From the "early transformation" stage the cultures progressed to a "complete transformation."[6]

As shown in Table 1, the time lapse between exposure to SV40 and transformation was much shorter for the three cultures of WI-26 substrain XXVI which transformed 3–5 weeks after infection, as compared to the XII substrain, which, when exposed at the level of the 27th passage during a period of active proliferation, transformed ten weeks after exposure to the virus.[6] Cultures of the parent WI-38

strain, which were also exposed at the time of their active proliferation (9th and 25th passages), transformed eight weeks after infection, whereas cultures of the parent and No. IV substrain, infected at the end of their *in vitro* lifetime, transformed after $4^1/_2$ weeks. The same holds true for D 90 and D 285 substrains of the W-5 CM culture which transformed $2^1/_2$–4 weeks after infection, in contrast to the parental culture which transformed nine weeks after exposure as a primary explant.[3, 6]

The "rapid" transformation process was also characterized by development of a much sharper contrast between the transformed and the nontransformed areas than noted in the "slow" transformation process. This was presumably observed because cells in the nontransformed areas were in a resting state in contrast to the areas of rapidly dividing transformed cells.

In the course of only one passage, subcultivation of the transforming cultures in the present series resulted in the establishment of virtually pure cultures of transformed cells, since presumably nontransformed cells were unable to divide.

Apart from these differences, the transformation process was morphologically identical, regardless of whether the cultures were infected by SV40 in a stage of active growth or in a stationary or regressive phase.

Following exposure to either of the two virus pools (see above), all transformed cultures became infectious. Results of preliminary titrations indicate that the yield of virus was greater from cultures which had undergone the "rapid" transformation process than from those having undergone "slow" transformation.

Chromosome studies: Detailed karyotype analysis of the WI-26 and WI-38 strains is the subject of a paper by Saksela and Moorhead.[2] In brief, chromosome counts of 200 WI-26 and 400 WI-38 cells of different substrains revealed no departure from classic diploidy *before* the level of the 37th–41st subcultures was reached. Thus, at the time of the earliest exposure to SV40 (27th passage for WI-26, XII and 9th for WI-38, P; Table 1), the cultures were chromosomally normal. During the declining phase of culture life, *after* the 40th passage, the WI-26 and WI-38 cells did show spontaneous aneuploidy characterized by various chromosomal aberrations in 2–30 per cent of the dividing cells, but monosomy of the 21–22 group was not a prominent feature of these abnormalities. None of the WI-26 and WI-38 substrains characterized by partial aneuploidy[2] and none of several other substrains observed during prolonged growth *in vitro* has undergone spontaneous transformation.

A karyotype of the cells of the buccal mucosa of patient W-5 was not determined. However, in the noninfected cultures at the level of the 7th subculture, 13 out of 58 cells examined were monosomic for a 21–22 chromosome.[3, 7] The proportion of monosomic cells increased during further cultivation, as indicated by the fact that 41 out of 47 cells at the level of the 17th–19th passages and all of the 16 cells examined at the level of the 20th passage were characterized by the absence of one chromosome from the 21–22 group.[7]

The cells of the W-5 substrains (Table 1) were also characterized by monosomy for the 21–22 chromosome, the proportions being about 80 per cent for D 90 and about 50 per cent for D 285, respectively.

Four weeks elapsed between the observed morphologic transformation after SV40 infection and the first appearance of high frequency of dicentrics, minutes, and other obvious chromosome changes in substrain XXVI of WI-26. The time period between these two events was identical to that observed in the "slow" transformation process of cultures infected in an actively proliferating phase.

Discussion.—The exact mechanism of the transformation process of tissue cultures by tumor-inducing viruses is essentially unknown. Therefore, it would be difficult to find an easy explanation for the unusually rapid transformation of human cell cultures infected with SV40 at the end of their *in vitro* lifetime, although it would be tempting to link together these two phenomena, the age of the culture and the "transformation time" (the time from exposure of the cultures to their morphologic alteration). Before this will be considered, however, thought should be given to the possibility that the low metabolic activity of cells unrelated to their age *in vitro* predisposes the culture to a more rapid transformation with SV40.

This hypothesis which stresses the physiologic state of the culture rather than the "genetic" makeup of its cell population can be put to test in SV40 transformation experiments in which proliferation of cells will be artificially inhibited during any phase of cultivation. However, the results of Shein and Enders[4] suggest a lack of relationship between the low metabolic activity of cells and the "transformation time."

If the low metabolic activity *per se* of cells in culture does not predispose them to a more rapid transformation, then the relationship between the process of aging *in vitro* and susceptibility to transformation with SV40 should be given more prominent consideration. Within this context it may be postulated that the target for SV40 transformation is a "defective" cell which is predominant in the cell population of a culture at the end of its lifetime, but represents only a small fraction of cells in an actively proliferating culture. This "defective" cell has a low capacity for proliferation and its "defectiveness" would be expressed as aneuploidy when such cells do undergo mitosis. Aneuploidy was prevalent in the aged cultures of WI-26 and WI-38 strains.[2] Infection with SV40 would restore the proliferative capacity of the "defective" cells and set in motion the process of transformation.[6] This fact is compatible with the known effect of infection with SV40, i.e., stimulation of mitosis either by direct action on the cell or by suppression of normally occurring mitotic inhibition. This characteristic activity, which has been observed in cultures of human and hamster fibroblasts prior to their morphologic transformation, is not limited to these normal cell systems since proliferation of HEp-2[8] and HeLa[8, 9] cells, and of polyoma virus-transformed hamster cells was also markedly enhanced after infection with SV40.[9]

If in actively proliferating cultures the number of "defective" cells is small, then the time between stimulation of the mitotic apparatus by infection with SV40 and proliferation of the infected cells to a point of formation of morphologically distinguishable colonies may be long. This may be particularly true if the growth potential of the "nontransforming" cells of the same culture is high, as the case may be for a "young" culture of human fibroblasts. Conversely, in aging cultures "defective" cells present in large number may need to undergo only a few cell

divisions after stimulation by SV40 infection before the morphological changes will be detected in the culture.

The role of the missing 21–22 chromosome in relation to the transformation process is difficult to evaluate. Deletion of this particular chromosome, one of the two small acrocentrics, has already been observed as an early change in the transformation process after infection with SV40 virus.[4, 7] It cannot yet be said whether this deletion is a specific phenomenon or occurs only as the result of a selective advantage exhibited by cells monosomic for a small acrocentric, as compared to cells in which the disturbance of karyotype is more drastic. Obviously, this monosomy is not a sufficient condition for transformation since many uninfected W-5 CM cultures have not transformed, although observed for a long period of time.

It is possible that "defective" cells are not present in primary explants but appear *in vitro* as the result of conditions leading to mutations among the proliferating cells (background irradiation, heterologous sera, etc.). The "defective" cell may also arise as the result of the presence of a "virus-like agent" which, on the one hand, may be indirectly responsible for the finite lifetime of cultures but, on the other hand, may facilitate transformation by SV40.

Interaction between SV40 and an "unknown" virus has few parallels in other animal virus systems except perhaps in the case of nodule-inducing (NIV) and mammary tumor viruses (MTV) which, as postulated by De Ome,[10] have to interact in order to cause mammary adeno-carcinoma in mice.

The difference between this system and the SV40-induced transformation lies in the fact that particles which may represent NIV were observed under electron microscopy,[10] whereas there is no known test for the presence of any virus which predisposes the mammalian cells to transformation by SV40.

The possibility cannot be excluded that the presence of such an agent can be demonstrated if SV40-infected actively proliferating cultures would transform more rapidly after exposure to extracts from "defective" cells or after previous cell-to-cell contact with their cultures.

Finally, it is possible, although not very probable, that infection of either aging or stationary cultures leads to selection of particles of SV40 endowed with a character for "rapid" transformation. This hypothesis is being tested by the exposure of actively proliferating cultures to preparations of virus obtained from cultures infected in a stationary phase. Results of experiments performed to date do not support this theory.

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*PROPERTIES OF A TEMPERATURE-SENSITIVE REGULATORY SYSTEM**

BY JONATHAN GALLANT AND ROSS STAPLETON

DEPARTMENT OF GENETICS, UNIVERSITY OF WASHINGTON, SEATTLE

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The synthesis of a number of inducible and repressible enzymes in bacteria has been shown to be controlled by the interaction of specific metabolites—inducers and "corepressors"—with specific, cytoplasmically diffused gene products which have been termed "aporepressors."[1] Little is known, however, about the nature of the postulated aporepressors, the molecular bases of their specificities, or the level(s) at which they intervene in the transcription of the information of structural genes.

In principle, some light might be shed on these questions through physiological experiments. Accordingly, numerous workers have examined the effect on enzyme repression of various regimes of unbalanced growth.[2] The ambiguities of these studies probably stem from two features of enzyme repression: (1) the synthesis of many inducible or repressible enzymes is subject to a complex and little understood repressive effect of carbon source dissimilation;[3] physiological manipulations which affect the pattern of catabolism consequently produce profound secondary effects on the rate of synthesis of regulated enzymes (e.g., the effect of thymine starvation on beta-galactosidase induction).[4] (2) If aporepressor molecules have a very high affinity for their sites of action[5] or a very high rate of synthesis,[6] then the sensitivity of the regulatory system may be too slight, or its response too transient, to be detectable. In particular, if aporepressors are rapidly synthesized and rapidly turned over, inhibitor studies would be complicated by the speed with which the steady state level of aporepressor will return to normal after the removal of inhibition.[6]

As a result of these considerations, we have sought a regulatory system in which the difficulties described above might be avoided. Such a system should: (1) be insensitive to catabolite repression, and (2) have a damaged regulatory system, hopefully one in which the rate of synthesis of aporepressor could be experimentally controlled. The synthesis of alkaline phosphatase in *E. coli* seems to satisfy condition (1), in that it is insensitive to catabolite repression[4, 7] while subject to a highly specific and genetically defined end product repression.[8] Consequently, we isolated a mutant which showed temperature-sensitive repression of alkaline phosphatase synthesis.[9] The present communication deals with the kinetics of phosphatase synthesis in this strain. Our observations indicate that the temperature sensitivity applies to the rate of synthesis of aporepressor. Variation in this rate